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Symposium

The Metabolism and Function of the Fat-Soluble Vitamins A, E and K

PRESENTED BY THE NATIONAL VITAMIN FOUNDATION

PART II OF TWO PARTS

Symposium on

the Metabolism and Function of the

Fat-Soluble Vitamins A, E and K

This Symposium was presented on November 7 and 8, 1960, at the University of Illinois, Urbana, Illinois, under the sponsorship of The National Vitamin Foundation, Inc., New York, New York.

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Symposium on the Metabolism and Function of the Fat-Soluble Vitamins A, E and K

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VOLUME NINE NUMBER FOUR, PART II

The Absorption of Beta-Carotene and Its Conversion into Vitamin A

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AT THE OUTSET, I wish to thank Dr. B. Conner Johnson of this University and the National Vitamin Foundation for their kind invitation to participate in this interesting symposium on the fat soluble vitamins. It is a real pleasure for me to visit the University of Illinois and to meet colleagues interested in the same challenging, if too often exasperating, problems of vitamin function and metabolism. My own studies have dealt wtih the earliest stages of vitamin A metabolism; namely, the conversion of β-carotene into vitamin A, and initially I wish to summarize some of the major findings in this field.

Studies on the absorption of β-carotene and on its conversion into vitamin A occupy such a wide place in the literature on the fat soluble vitamins that one might fully occupy himself by merely pondering the thoughts and extensive efforts of others. Fortunately—and doubly so for a relative newcomer to vitamin research such as myself—these problems have been examined in several valuable surveys, by Moore[1] in his excellent monograph on vitamin A, by Deuel[2] in his encyclopedic volumes on the lipids, and in several recent reviews.[3,4]

Major considerations might be briefly summarized as follows: β-carotene in low doses is fairly well absorbed in the intestine, but at higher doses appears in the feces in large amounts. Since β-carotene is lipophilic, an emulsifying agent—and the natural emulsifier is bile—is required for its absorption. Fats in the diet enhance β-carotene absorption, perhaps by stimulating bile flow, and antioxidants in small amounts also seem to do so, probably by preventing the destruction of β-carotene in the lumen. Differences seem to exist in the absorption rate with different carotenoids, and inhibition of β-carotene absorption by paraffin and by other carotenoids has been observed.[5] The integrity of the mucosa is necessary for absorption to occur.

With respect to vitamin A, the rate of absorption from the intestine is rapid compared with uptake of β-carotene, and its absorption is less dependent on the presence of bile. Antioxidants, dietary fat and detergents all enhance absorption.

Since the demonstrations by von Euler[6] and Moore[7] that β-carotene relieved vitamin A deficiency in rats, the mechanism of its conversion into vitamin A has been a subject of intense interest. Throughout the years two major mechanisms have been defined. The first is the central cleavage of β-carotene into two molecules of vitamin A, initially proposed by Karrer[8] and modified by Hunter[9] to include retinene as an intermediate. Evidence supporting this suggestion is that α-carotene, γ-carotene, and other asymmetric compounds with but one β-ionone ring had 50 per cent or less the activity of β-carotene in growth tests. Furthermore, under carefully defined conditions of β-carotene and α-tocopherol dosage,[10,11] β-carotene was twice as effective as vitamin A, mole for mole, in stimulating the growth of vitamin A deficient rats. On the other hand, in many nutritional studies[12] β-carotene seemed to be no more effective, mole for mole, than vitamin A, and with the extensive work on the relative potencies of carotenoid isomers by Deuel, Zechmeister, and their colleagues,[13] it became evident that the relative biologic values of various

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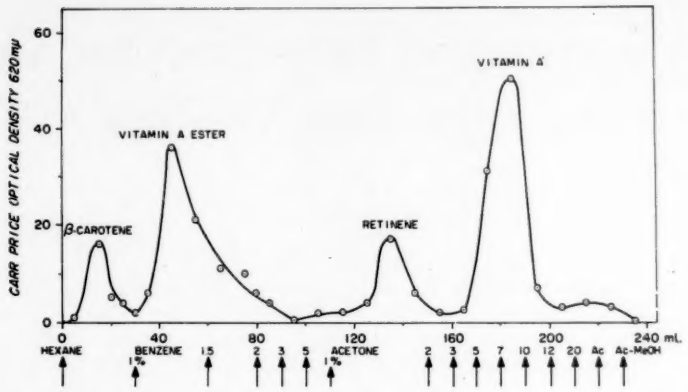

FIG. 1. The separation of β-carotene and vitamin A derivatives on deactivated alumina containing 6% H_2O (v/w).

carotenoids were not clustered about 0 and 50 per cent, but rather were spread regularly from 0 to 60 per cent of β-carotene potency.

It was of great interest, therefore, when Glover and Redfearn[14] suggested that one molecule of vitamin A might be formed from one molecule of β-carotene by a stepwise oxidation of β-carotene from one end of the conjugated central chain. Glover and his colleagues[15,16] found that β-apo-carotenals and β-apo-carotenoic acids, which possessed but one β-ionone ring, were biologically active in stimulating the growth of deficient rats, and that radioactive acidic products as well as vitamin A were present twenty-four hours after radioactive β-carotene was fed to rats. Kraus[17] reported similar results with radioactive β-carotene.

All these studies were conducted with intact animals, and consequently many of the deductions about the absorption and cleavage of β-carotene were based by necessity on observations which were only indirectly related to the process being studied. This is particularly true of biologic methods. For example, the growth response to a dose of substrate depends not only on the stoichiometry of cleavage, but also on the relative stability, absorption rate, transport, uptake by tissues, rate of turnover, metabolism, and ultimately when and how long, where and how much, what compound is needed to give a maximal growth response. Change in the concentrations of β-carotene and vitamin A in plasma, the vaginal smear response, liver storage of vitamin A, differences between fecal and ingested carotenoids, etc., result from changes in several physiologic processes rather than a single one; and hence, it is not surprising that considerable controversy exists regarding the influence of various factors on β-carotene absorption and cleavage.

Chemical and physical methods which have been used extensively also suffer from shortcomings. For example, the Carr-Price reaction and changes in spectra upon irradiation are relatively nonspecific, and this lack of specificity becomes critical when small amounts of vitamin A must be detected in the presence of large amounts of β-carotene and its oxidation products. Unfortunately, this is the precise situation that one encounters in studying the conversion of β-carotene into vitamin A. Thompson and his colleagues[18,19] fed milligrams of β-carotene to the rat and found a few micrograms of vitamin A in the intestinal mucosa, a conversion yield of less than 5 parts per 1000. Similar minimal rates of conversion might be calculated from a consideration of the daily nutritional requirements of animals. For example, if a rat requires 3 μg. of vitamin A per 100 gm. per day for maximum life maintenance,[20] a minimal but fully adequate conversion rate for a 200 gm. rat on a diet containing carotene but free of vitamin A would be 0.25 μg. of vitamin A per hour. In view of these considerations, it is no wonder that various reports appeared in which the conversion of β-carotene into vitamin A *in vitro* was claimed on the basis of conventional

methods for vitamin A analysis. Bieri and Pollard,[21] however, in an exemplary reexamination of the methods employed in these studies, were unable to confirm them.

I would like to deal with several aspects of the problem of β-carotene absorption and its conversion into vitamin A as follows:

1. The development of methods for the specific detection of small quantities (about 0.05 μg.) of vitamin A in the presence of β-carotene and its oxidation products.
2. The kinetics of the absorption and cleavage of β-carotene *in vivo*.
3. The requirement for bile acids in the absorption of β-carotene and its cleavage into vitamin A, *in vivo* and *in vitro*.
4. Other requirements for β-carotene cleavage.
5. Some observations on the mechanism of cleavage.

METHODS FOR THE SPECIFIC DETECTION OF SMALL AMOUNTS OF VITAMIN A

Our methods were based on the use of radioactive β-carotene of relatively high specific activity (ca. 1000 c.p.m./μg.). Radioactive C^{14} β-carotene was prepared biosynthetically in two ways: (1) by growing the photosynthetic algae Chlorella pyrenoidosa on $C^{14}O_2$ as a sole carbon source,[22] and (2) by adding 1-C^{14} acetate to a growing culture of Phycomyces blakesleeanus.[23] The carotenoids were extracted by grinding repeatedly with ether-ethanol, and the nonsaponifiable fraction in hexane was chromatographed on Woelm alumina, grade 1. The radioactive β-carotene fraction was recrystallized to constant specific activity and mixed with crystalline nonradioactive β-carotene to yield a preparation with a specific activity of about 1,000 c.p.m. per μg.[24] The elution pattern for β-carotene and vitamin A derivatives from columns of deactivated alumina is shown in Figure 1. The separation of β-carotene from vitamin A ester on these columns is adequate, but may be improved by using hexane as the eluant for a longer period.

The methods which have been used throughout this study[24,25] are summarized in Figure 2. In general, clear dispersions of β-carotene in 1 to 5 per cent Tween 20 were employed for all experiments. Isolation procedures were carried out rapidly in dim light and at temperatures below 60° c. Since radioactive oxidation products of β-carotene contaminated all of the fractions from alumina columns, further characterization of vitamin A derivatives was required. Therefore, the combined vitamin A ester fractions were saponified to yield vitamin A alcohol, vitamin A alcohol was oxidized with MnO_2 to retinene, and finally the semicarbazones of retinene were prepared. Artifacts were eliminated by employing this procedure, and the specific activity of subsequent derivatives remained constant.

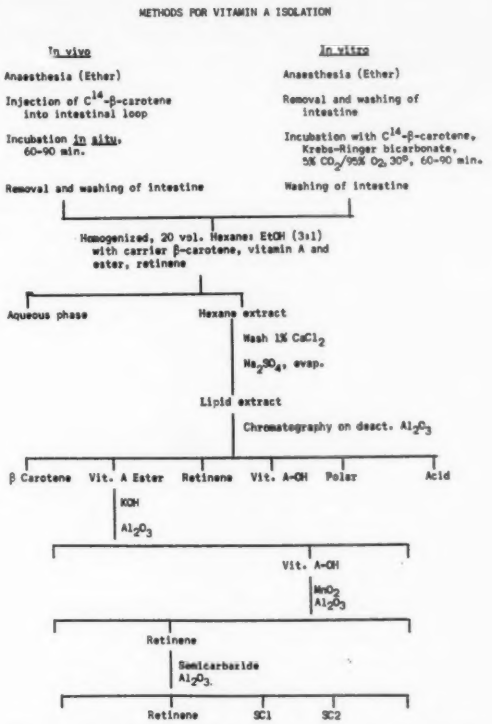

FIG. 2. A flow sheet of the methods employed for the isolation of vitamin A derivatives from incubations with C^{14}β-carotene *in vivo* and *in vitro*. SC1 and SC2 are the two retinene semicarbazones.

KINETICS OF THE ABSORPTION AND CLEAVAGE OF β-CAROTENE IN VIVO

The absorption of small doses of β-carotene from the lumen of an intestinal loop followed first-order kinetics[24] as shown in Figure 3. The theoretical curve for a first order rate constant of 0.83 $hour^{-1}$ as well as the exper-

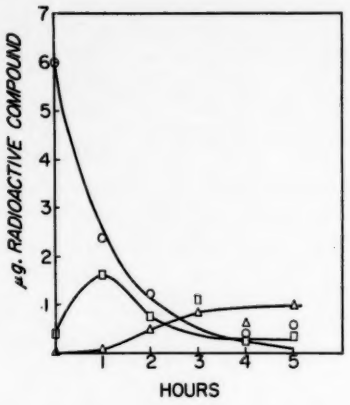

FIG. 3. Radioactivity in the intestinal lumen, intestinal wall and liver at various times following the intraduodenal injection of 8 μg. of C^{14} β-carotene. The μg. radioactivity are given for the intestinal contents (○), intestinal wall and mucosa (□) and liver (△). (From: OLSON, J. A. *J. Biol. Chem.*, 263: 349, 1961.[24])

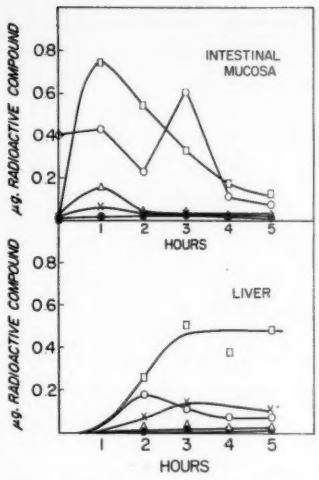

FIG. 4. Distribution of radioactivity in chromatographic fractions of the intestinal wall and liver following the intraduodenal injection of 8 μg. of C^{14} β-carotene: β-carotene (○), vitamin A ester (□), retinene (△), vitamin A alcohol (×), and the terminal polar fraction (●). (From: OLSON, J. A. *J. Biol. Chem.*, 263: 349, 1961.[24])

imental points are plotted. The total radioactivity in the intestinal mucosa reached a peak at one hour and then decreased, whereas radioactivity was first detectable in the liver at one hour and reached a plateau in three to five hours. Thereafter, the three extracts were separated into various components by chromatography. In the lumen extract, β-carotene and its oxidation products were found, but vitamin A derivatives could not be detected. The major vitamin A derivative of the intestinal mucosa at all times was vitamin A ester (Fig. 4). Small amounts of retinene and vitamin A alcohol were present at one hour, but diminished thereafter. The polar fraction was practically devoid of radioactivity at all times. Attention should be called to the β-carotene content of intestinal mucosal extracts. Its value was high in one minute incubations and did not vary in a consistent way with time. This suggested that β-carotene is bound in a nonspecific manner on the mucosal surface, and that most of the bound β-carotene is not directly related to β-carotene absorption or to its cleavage into vitamin A. Other evidence supports this interpretation, namely:

1. Bound β-carotene is only a fraction of the vitamin A present in the mucosa of the best experiments *in vivo*, but increases as the integrity of the mucosa declines.

2. Intestines which are poisoned with mercuric chloride or with cyanide, or which are incubated anaerobically, bind large amounts of β-carotene but are unable to catalyze vitamin A formation.

Thus, the amount of β-carotene in mucosal extracts could not be equated with the quantity of *intracellular* β-carotene, and the absorption process could not be differentiated experimentally from the cleavage reactions. On the other hand, the over-all process of β-carotene absorption and cleavage was adequately measured by the amount of vitamin A ester formed in the mucosa in one hour under standard conditions.

In the liver (Fig. 4), vitamin A ester again was the major component, vitamin A alcohol was present in reasonable amounts, but retinene and the terminal polar fraction were not radioactive. The concentration of β-carotene was maximal at two hours, and then diminished.

The rate of vitamin A ester formation from β-carotene in the above experiment (0.7 μg./hour) was considerably lower than reported values.[18,19] It seemed reasonable that the rate would depend on the amount of β-carotene given, and this was shown to be the case[24]

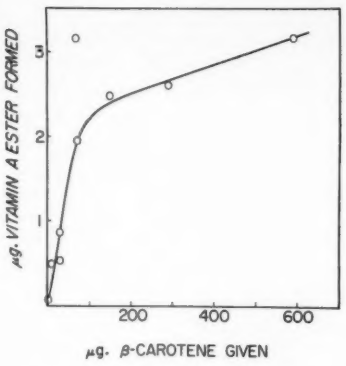

FIG. 5. The relation of the dosage of β-carotene to the amount of vitamin A ester formed in the intestinal mucosa during a sixty minute incubation *in vivo*. (From: OLSON, J. A. *J. Biol. Chem.*, 263: 349, 1961.[24]).

(Fig. 5). Rates as high as 5 μg. of vitamin A ester per hour were observed in some instances. It is interesting that vitamin A ester formation became less dependent on the dosage at β-carotene levels above 100 μg.

ABSORPTION OF β-CAROTENE AND ITS CLEAVAGE INTO VITAMIN A, IN VIVO, AND IN VITRO

The Requirements for Bile Acids

The role of various secretions in promoting β-carotene absorption and cleavage *in vivo* was then assessed. The addition of an extract of gastric mucosa had no effect on normal uptake and vitamin A ester formation, whereas vitamin A ester was not formed when the intestinal loop was washed out and the bile duct was ligated[24,25] (Table I). In bile duct ligated animals, the requirement for bile was satisfied completely by glycocholic acid (Table II). Cholic acid was somewhat less effective, and 5 per cent deoxycholic destroyed the mucosa and allowed no vitamin A formation.[24]

The requirement for bile is not surprising and has been shown in the past, but in this case its demonstration has a novel twist: namely, the β-carotene administered was solubilized in a clear Tween 20 dispersion rather than in arachis oil or in some other lipid vehicle. One of my students, Mr. Zachman, in connection with a careful study of the enzymatic reduction of retinene,[26] has found that the anhydrous micelle particle size of Tween 80, when studied by ultracentrifugal analysis,

TABLE I

Effect of Intestinal Factors on Vitamin A Ester Formation from C^{14} β-Carotene in Washed Ligated Rat Intestine (*in Situ*)

Experiment No.	Condition	Vitamin A Ester Formed (μg.)
35a	Normal	0.80
35b	+ Gastric mucosal extract	0.77
36a	Normal	0.16
36b	Bile and pancreatic duct ligated	0.01
36c	Bile duct ligated	0.02

TABLE II

Effect of Bile and Bile Acids on Vitamin A Ester Formation in Bile Duct-Ligated Rats

Experiment No.	Suspension	Vitamin A Ester Formed (μg.)
II-38	Rat bile	0.68
	5% Glycocholate + 5% Tween 20	0.66
	Rat bile + 5% Tween 20	0.57
	5% Tween 20	0.04
II-104	5% Glycocholate + 5% Tween 20	0.74
	5% Cholate + 5%Tween 20	0.65
	5% Deoxycholate + 5% Tween 20	0.03
	5% Tween 20	0.08

NOTE: β-Carotene (10,000 c.p.m. in 10 μg.) was injected intraduodenally in 1 ml. of the indicated suspension. Experimental period was sixty minutes.

is about 100,000. Hence, these micelle particles are much smaller than 0.5 μ, the maximal size defined for the absorption of lipid particles.[27] It is clear, therefore, that bile does not function merely as a general emulsifying agent in this case, but must have some more specific action. Efforts were made to define the nature of this effect in greater detail by using *in vitro* systems.

When washed, longitudinally cut sections of intestine were incubated in Krebs-Ringer bicarbonate buffer at 30°c. under 5 per cent CO_2-95 per cent O_2, vitamin A ester was produced from β-carotene.[25] This conversion was dependent in a fairly critical way on the con-

TABLE III

Effect of Synthetic Glycocholate and Taurocholate on Vitamin A Ester Formation

Concentration (%)	Glycocholate	Taurocholate
0	0.10	0.01
0.2	0.24	0.13
0.4	0.46	0.31
0.6	0.29	0.25
0.8	...	0.07
1.0	0.20	0.08

NOTE: Intestinal sections were incubated with 4 ml. of 1 per cent Tween 20-Krebs Ringer bicarbonate buffer containing 20 μg. β-carotene (1,000 c.p.m./μg.) and the given concentrations of conjugated bile salts at 30°c. for one hour in a Dubnoff shaker under 5 per cent CO_2–95 per cent O_2.

TABLE IV

Effect of Various Anionic Detergents on Vitamin A Ester Formation from β-Carotene *in Vitro*

Detergent	Vitamin A Ester Formed (μg.)
0.3% Glycocholate	0.24
0.3% Cholate	0.10
0.3% Oleate	0.04
0.3% Cephalin	0.04
0.3% Deoxycholate	0.02

NOTE: Intestinal sections were incubated with 4 ml. of 1 per cent Tween 20-Krebs Ringer bicarbonate buffer containing 10 μg. β-carotene (1,000 c.p.m./μg.) and the given detergent at 30°c. for one hour in a Dubnoff shaker under 5 per cent CO_2–95 per cent O_2.

centration of bile salt present (Table III). Both taurocholate and glycocholate were most active at 0.4 per cent. In these instances, the conjugated salts were synthesized by the ethyl chloroformate method[28] and were chromatographically pure.[29] A commercial preparation of glycocholate, which contained several chromatographic components, gave similar results.

Two aspects of the bile salt requirement were considered; firstly, the effect of bile acid structure, and secondly, the nature of the substrate absorbed. Bile acids and their amino acid conjugates bear a negative charge. Other negatively charged substances, such as oleic acid and cephalin (phosphatidyl serine) however, were inactive (Table IV). The importance of the N acyl bond was also evaluated by testing N-palmitoyl and N-myristoyl derivatives of glycine and alanine. None of these compounds enhanced the conversion. Furthermore, lithocholic acid was ineffective, and its conjugates had only slight activity. It is important to note that the mucosal surface was adversely affected by lithocholate and its derivatives.

TABLE V

Effect of Glycocholate on the Uptake and Esterification of Vitamin A Alcohol *in Vitro*

Glycocholate Concentration (%)	Vitamin A Ester Formed	
	Experiment No. 77 (μg.)	Experiment No. 69 (μg.)
0	2.16	3.63
0.2	1.60	3.40
0.4	2.06	3.50
0.8	...	0.52
1.0	0.69	...

NOTE: Intestinal sections were incubated with 4 ml. of 1 per cent Tween 20-Krebs Ringer bicarbonate buffer containing 12 μg. (experiment no. 77) or 20 μg. (experiment no. 69) of C^{14} vitamin A alcohol (900 c.p.m./μg.) at 30°c. for one hour in a Dubnoff shaker under 5 per cent CO_2–95 per cent O_2.

With respect to other substrates, the uptake and esterification of C^{14}-vitamin A alcohol was not dependent on the presence of glycocholate[25] (Table V). Similarly, the conversion of C^{14} retinene to vitamin A ester proceeded well in the absence of glycocholate. Nor did the uptake and esterification of vitamin A acid seemingly require glycocholate (Table VI). In the latter case, it is difficult to ascertain how much vitamin A acid was bound nonspecifically on the surface of the mucosa, and how much was intracellular. The total amount in the lipid extract was unaffected by glycocholate, however, and ester formation, although small, was unaffected or perhaps even depressed by the presence of glycocholate.

The major findings might be summarized as follows: β-carotene requires conjugated bile salts for its conversion into vitamin A ester, whereas the formation of vitamin A ester from vitamin A or retinene, and the esterification of vitamin A acid do not. The stimulation of β-carotene cleavage is not due simply to the negative charge on the micelle

TABLE VI
Uptake of Vitamin A Acid by Intestinal Sections *in Vitro*

Fraction	Eluant	No. of Experiments	Control (c.p.m.)	With Glycocholate (c.p.m.)
Lipid extract	...	2	22,300	21,000
Alumina fractions				
Pre-ester	Hexane	2	25	25
Ester	Hexane	2	740	180
Ret-Vitamin A	20% Acetone	2	0	0
Polar	Acetone	2	140	80
Acid	Acetic Acid	2	8,000+	11,460+

NOTE: Intestinal sections were incubated in 4 ml. of 1 per cent Tween 20-Krebs Ringer bicarbonate buffer containing 2 μg. vitamin A acid (20,000 c.p.m./μg.) with or without 0.4 per cent glycocholate at 30° for one hour in a Dubnoff shaker under 5 per cent CO_2-95 per cent O_2.

TABLE VII
Effect of Conjugated Bile Salts on Synthetic Reactions of Intestinal Sections Incubated *In Vitro*

Substrate and Specific Activity	Total Amount of Substrate (μg.)	Isolated Product	No. of Experiments	Control (c.p.m.)	With 0.4 Per Cent Glycocholate (c.p.m.)
2-C^{14} leucine 4.5×10^3 c.p.m./μg.	400	Protein	3	20,200 ± 4,100	11,600 ± 5,000
2-C^{14} leucine 4.5×10^3 c.p.m./μg.	400	Protein	2	32,000 ± 35	37,000* ± 6,600
1-C^{14} acetate		Nonsaponifiable fraction	3	5,370	7,060
1.8×10^4 c.p.m./μg.	64	Nonvolatile fatty acids	3	22,250	18,130
U-C^{14} glucose	4,000	Total lipid	3	280	370
6.0 c.p.m./μg.		Triglyceride fraction		170	200
U-C^{14} glucose	12.5	Total lipid	3	3,040	3,320
2.0×10^3 c.p.m./μg.		Triglyceride fraction		1,760	1,590

* With 0.4 per cent taurocholate instead of glycocholate.

NOTE: Intestinal sections were incubated in 4 ml. of 1 per cent Tween 20-Krebs Ringer bicarbonate solution containing 1 mg. of glucose per ml. (except as indicated above) and the given substrate at 30° for one hour in a Dubnoff shaker under 5 per cent CO_2-95 per cent O_2. Protein was precipitated from intestinal homogenates with 10 per cent trichloroacetic acid, was extracted thoroughly with hot ether:ethanol (1:1), and a small known fraction of it was plated evenly on an aluminum planchet and counted. The separation of the nonsaponifiable and nonvolatile fatty acid fractions of intestine after incubation with C^{14} acetate was carried out by usual procedures. Saponification was not employed in the glucose studies. The lipid extracts were chromatographed on alumina, and the triglyceride fraction was separated and counted.

or the N-acyl bonding, but is some property of the cholic acid molecule. Other bile acids tested might be inactive because they lack specific structural characteristics, or because of their destructive action on the intestinal mucosa.

These observations on the relative effectiveness of bile acids in stimulating the absorption of lipid substances is not unique with β-carotene. Treadwell and his colleagues[30] found that the absorption of cholesterol and its appearance in the lymph of thoracic duct cannulated animals was a function of bile acid structure. And recently, Dawson and Isselbacher[31] found similar requirements for the esterification of palmitic acid by intestinal sections of the rat *in vitro*. In many respects their data parallel ours with β-carotene. They made, however, one very interesting ancillary observation: namely, that the conversion of glucose to triglyceride glycerol is stimulated fourfold by taurocholic acid. They suggest that glycocholate *might* stimulate synthetic reactions within the mucosa, and hence that the enhanced absorption of palmitic acid might be a secondary result of this stimulation.

TABLE VIII

Recovery of β-Carotene and Vitamin A Derivatives from Intestinal Loops of the Rat *in Situ*

Fraction	Radioactivity (μg.)		
	Experimental	Control	Difference
Lipid extract	14.3	18.5	−4.2
Alumina column fractions			
β-carotene	11.3	15.6	−4.3
Vitamin A ester	2.0	0.2	+1.8
Retinene	0.3	0.4	−0.1
Vitamin A alcohol	0.5	0.4	+0.1
Terminal polar	0.1	0.2	−0.1

NOTE: β-Carotene (37,600 c.p.m. in 23.5 μg.), suspended in 1 ml. of 5 per cent Tween 20 + 5 per cent glycocholate, was injected into a washed ligated loop of rat intestine *in situ*. The control experiment was similarly conducted except that the bile duct was ligated and no glycocholate was included in the β-carotene suspension. The experimental period was one hour. The lumen contents and the wall were analyzed together. (From: OLSON, J. A. *J. Biol. Chem.*, 236: 349, 1961.[24])

TABLE IX

Formation of Radioactive Acidic Products during β-Carotene Cleavage into Vitamin A by Intestinal Loops *in Vivo*

Fraction	Radioactive Products (μg.)
Alumina column fractions	
β-carotene	0.52, 0.45
Vitamin A ester	1.02, 1.07
Retinene, Vitamin A, Terminal polar	0.15, 0.09
Aqueous phase	
Alkaline ether extract	0.02, 0.02
Acidic ether extract	0.11, 0.27

NOTE: β-Carotene (16,000 c.p.m. in 20 μg.), suspended in 1 ml. of 5 per cent Tween 20, was injected into a ligated intestinal loop *in situ*. The experimental period was one hour. The ethanolic aqueous phase resulting from separation of the hexane phase was rendered basic and extracted thrice with ether. Thereafter the aqueous solution was acidified and extracted continuously with ether for twelve hours. Aliquots of the washed ether extracts were plated and counted. The results of two experiments are given.

Since our own results point rather to a primary action of bile salts on the absorption of β-carotene, we investigated the effect of glycocholate on several major synthetic pathways in intestinal sections: the formation of protein from leucine, of sterols and fatty acids from acetate, and of neutral lipid from glucose. The results are given in Table VII. In our hands, glycocholate had no major stimulatory effect on these synthetic pathways. I wish to add, however, that the conditions employed by Dawson and Isselbacher differed from ours in several ways, and hence that we are not necessarily at odds. The important point here is that the rate of conversion of β-carotene into vitamin A was maximally stimulated by conditions which had no apparent effect on several synthetic pathways.

Other Requirements for β-Carotene Cleavage

Oxygen is essential for the conversion of β-carotene into vitamin A ester. When intestinal sections were incubated under 5 per cent CO_2-95 per cent N_2, a considerable amount of β-carotene was bound to the mucosal surface but no vitamin A ester was found. Although calcium and magnesium seem to play a role in several absorptive processes, the removal of these metals from the medium had no effect on vitamin A ester formation. Furthermore, treatment of the intestinal sections with ethylene diamine tetra-acetate did not inhibit the formation of vitamin A ester.

OBSERVATIONS ON THE MECHANISM OF β-CAROTENE CLEAVAGE

If β-carotene is cleaved at its central double bond into two molecules of vitamin A, then one might expect to find all of the radioactivity disappearing from β-carotene in vitamin A derivatives. On the other hand, if stepwise cleavage of β-carotene into one molecule of vitamin A is the predominant pathway, only half the radioactivity disappearing from β-carotene would be found in vitamin A, and the remainder would be present largely in acidic products. Attempts were made, therefore, to conduct careful balance experiments in the hope of resolving this problem. Unfortunately, the over-all yields were not high enough to permit precise definition of the major pathway of cleavage. Nonetheless, some typical results[24] are given in Table VIII. The recovery of radioactivity was 80 per cent of the β-carotene injected into the control loop, but was only 60 per cent of that injected into the experimental

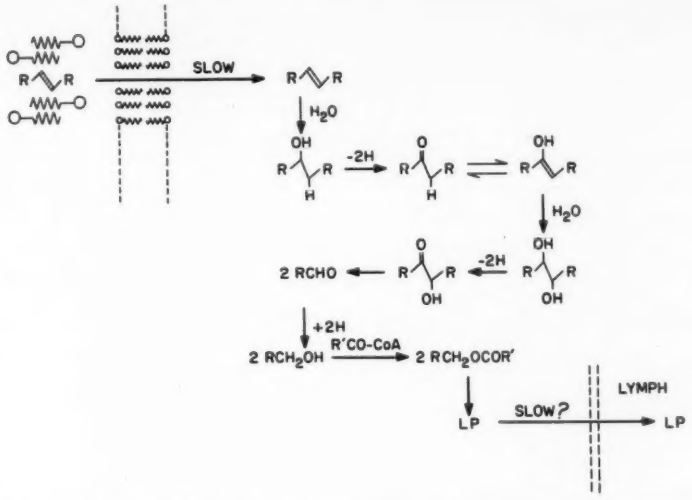

FIG. 6. A postulated pathway for the absorption of β-carotene and its cleavage into vitamin A. (From: OLSON, J. A. *J. Biol. Chem.*, 263:349, 1961.[34])

loop. After chromatographic separation, the difference in β-carotene between the two experiments was 4.3 μg., whereas the difference in vitamin A ester was 1.8 μg., or about 40 per cent of the β-carotene difference. For several reasons, however, this percentage must be considered a minimal value. Firstly, the total difference between experimental and control intestines is probably due to material transported out of the intestinal system, and this material would largely be vitamin A ester. Secondly, vitamin A is metabolized slowly by the mucosa to other products, and hence any such compounds would not be measured as vitamin A in these experiments.

In addition, the radioactivity in acidic fractions of the intestine after incubation with C^{14}-β-carotene was measured. As shown in Table IX, the acidic fraction contained less than 25 per cent of the radioactivity in the vitamin A ester fraction. Other experiments, in which the washed intestine was saponified, yielded similar results.

Although these experiments are not conclusive, they favor a central cleavage mechanism as the major pathway for β-carotene metabolism in the intestinal mucosa. The arguments are that any reasonable correction of the observed 40 per cent conversion value would make it greater than 50 per cent, the maximal amount possible if the stepwise cleavage mechanism were solely operative, and that appreciable amounts of acidic and polar products, which would be expected from stepwise cleavage, did not accumulate in the mucosa.

SUMMARY

The final diagram[24] (Fig. 6) may be used to summarize our present thoughts on this subject. β-Carotene is absorbed from the intestine and is converted into vitamin A only in the presence of bile acids. Conjugated bile acids do not function solely in dispersing the lipophilic substrate into a micellar form which is suitable for absorption. Rather, they possess a more specific function which is lodged mainly in the cholanic acid structure, but is enhanced by conjugation. Cholic acid derivatives were most effect in stimulating the overall conversion. The presence of a negative charge or of an N-acyl glycine derivative in the micelle did not in itself enhance β-carotene cleavage. Since the uptake of vitamin A alcohol, retinene, and apparently of vitamin A acid was unaffected by conjugated bile acids, and since no apparent stimulation of intracellular synthetic reactions took place in their presence, it is likely that the bile salts act primarily by enhancing the absorption of β-carotene rather than by stimulating β-carotene cleavage. The action of bile salts in

molecular terms, however, is completely unknown.

The rate limiting step in the over-all conversion process also seems to reside at the absorptive step. If correction is made for the β-carotene bound nonspecifically—a binding which is independent of oxygen, occurs rapidly, and increases as the integrity of the intestinal preparation declines—the calculated amount of intracellular β-carotene is very low. Furthermore, the rate of uptake and esterification of vitamin A alcohol and of retinene is rapid compared to the rate of β-carotene conversion.

Few intermediates accumulate between β-carotene and vitamin A ester. Some retinene and vitamin A alcohol are present, and some nonpolar and acidic products form. The amount of these is small, however, compared to the amount of ester present; and there is no reason to believe that the acidic and polar compounds are intermediates of β-carotene cleavage. Indeed, no real evidence exists that retinene is a direct intermediate, although it is present in the intestine, reaches a maximal concentration early, and is converted to vitamin A ester. The requirement for oxygen may be involved in absorption, cleavage, or in the general maintenance of the tissue.

With reservations, a central cleavage mechanism seems to be favored by our results. A more detailed possible mechanism is outlined in Figure 6, based in large part on analogous reactions carried out by known enzyme systems. Only the reduction of retinene and esterification of vitamin A have been demonstrated thus far in cell free systems. Finally, the rise in vitamin A ester to a maximum followed by a decrease suggests that the formation of lipoprotein and its secretion into the lymph is a second rate-limiting step.

ACKNOWLEDGMENT

I am indebted to Mrs. Jean S. Herron for excellent technical assistance, to Hoffmann LaRoche and Company, Basle, for generous gifts of 6,7 C^{14}-vitamin A acetate and C^{14}-vitamin A acid, to Dr. Elwood Jensen for pure samples of lithocholic acid and its conjugates, and to the Atlas Powder Company for ample samples of various Tween detergents.

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DISCUSSION

DR. P. T. VARANDANI (*Urbana, Illinois*): For a long time workers in the vitamin A field have wondered about the functional form of vitamin A. Is it vitamin A *per se*, or is it a derivative which is the active form in the body? When a vitamin A-free diet is fed to animals, the reserve stores of the vitamin in the liver are exhausted considerably before actual deficiency symptoms appear. Conversely, when vitamin A is fed to a deficient animal at least 100 I.U. of vitamin A disappear before the storage begins. It appears that vitamin A is transformed in the body to a substance which functions in the maintenance of epithelial tissues and in the promotion of growth. After the proper level of this derivative is reached, vitamin A itself appears in the blood and finally is stored in the liver. We have obtained in our laboratory some evidence in support of such a hypothesis.

TABLE I

Subcellular Distribution of Vitamin A-2-C^{14} Acetate*

Fraction	Total in Cells (%)
Nuclei	10.8
Mitochondria	40
Microsomes	23
Supernatant fraction	32

* Total activity injected 2.64 μc.; divided into three doses given over a period of thirty-six hours.

The results in Table I show the subcellular distribution of radioactivity in the liver following the intraperitoneal injection of 2-C^{14}-labeled vitamin A ester into normal rats. Radioactivity appeared in all fractions; however, it can be seen from Table II that, whereas in the case of the mitochondrial and microsomal fractions all the activity is ether-extractable and therefore probably free vitamin A, in the case of the supernatant fraction much of the activity was ether-extractable only after treatment with KOH. This represents protein-bound activity and may be a functional metabolite of vitamin A.[1]

Wright[2] extended these experiments by injecting small amounts of vitamin A 1′,9-C^{14} (generously supplied by Hoffman-La Roche, Inc.) to a deficient rat. Most of the radioactivity in the organs (Table III) was bound to lipoprotein (released by alcohol treatment) or to protein by covalent bonds (released by alkali treatment).

In view of the involvement of vitamin A in mucopolysaccharide biosynthesis it is of interest to note that almost all the activity in colon is bound to protein.[3] Varandani et al.[4] obtained similar results when papain

TABLE II

Radioactivity of Subcellular Fractions Following Vitamin A-2-C^{14} Injection

Fraction	Ether Extractable (c.p.m.)	Ether Extractable after Alcohol Treatment (c.p.m.)	Ether Extractable after KOH Treatment (c.p.m.)
Supernatant	205,600 (61)*	47,400 (16)	34,000 (23)
Mitochondria	354,000 (97)	1,700 (0.3)	9,000 (2.7)
Microsomes	190,000 (93)	8,000 (4)	6,290 (3)

* These figures in parentheses indicate the per cent of total radioactivity of each subcellular fraction.

TABLE III

Distribution of Radioactivity Derived From Vitamin A-1′,9-C^{14} Acetate After Injection Into Depleted Rats*

Organ	Experiment	Total Activity (d.p.m.)	Ether Extractable (d.p.m.)	Ether Extractable	
				After Alcohol Treatment (d.p.m.)	After Alkali Treatment (d.p.m.)
Liver	1	27,150	2,640	10,700	13,800
Kidneys	1	6,800	1,070	3,160	2,570
Colon	1	1,720	None	595	1,125
	2	5,040	360	1,230	3,450
Heart	1	403	None	286	117
Adrenals	1	None	...	...	...
	2	2,480	None	2,600	None

* In experiment 1, 1.1×10^6 d.p.m. (75 μg.) were injected four hours prior to sacrifice; in experiment 2, 3.3×10^6 d.p.m. (225 μg.) were injected twenty hours prior to sacrifice.

was used in place of alkali. In summary, there appears to be a protein-bound vitamin A derivative in the body which may be a functional form of vitamin A.

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The Effect of Vitamin A Deficiency and Estrogen on the Uterus

WALTER J. BO, PH.D.*

IT IS WELL known that a deficiency of vitamin A and also overstimulation with estrogen causes a keratinizing metaplasia of the epithelium of the rat uterus. Wolbach and Howe[1] were the first to carry out detailed studies of the morphologic changes produced by the withdrawl of vitamin A from experimental animals. They observed that stratified squamous keratinizing metaplasia developed in all types of epithelia through the following sequence of histologic changes: atrophy of the original epithelium, proliferation of the basal cells which displaced the original epithelium and finally a differentiation into a stratified squamous keratinizing epithelium. In the rat, the uterine changes were first observed to occur in the endometrial glands. Metaplasia of the epithelium of the uterus of the guinea pig is more marked than for the rat.[2] In vitamin A deficient mice metaplasia of the uterine epithelium has not been observed to occur.[3]

Selye, Thompson and Collip[4] were the first among many investigators[5–14] to observe that prolonged treatment with estrogen produced metaplasia of the epithelium of the uterus. Although all authors agree that metaplasia of the uterine epithelium can be induced in experimental animals by overstimulation with estrogen, there is no agreement about the manner of origin and development of the abnormal epithelium. Loeb, Suntzeff and Burns[9] believe that in the mouse true metaplasia of the uterine epithelium may occur. However, they suggested that in most instances the stratified squamous epithelium which appeared in the uteri of estrogen-treated mice had been produced by a forward extension of the stratified epithelium of the cervix. They believed that the foward extension of the epithelium of the cervix into the horns of the uterus was preceded by ulceration of the mucosa of the uterus.

Fluhmann[15,16] reported that stratified squamous epithelium may be produced in the uterus of estrogen-treated rats by two separate and distinct methods, depending upon the epithelium involved. First, by a process he has called prosoplasia, the cervicovaginal epithelium is converted into a stratified squamous epithelium. This he believes occurs as a direct response to hormone treatment. Second, he believes that squamous metaplasia of the cornua of the rat uterus is preceded by extensive degeneration of the uterine epithelium. During the process of degeneration some of the cells survive and form plaques of squamous epithelium which, after growth and extension, form a continuous stratified squamous epithelium. The above author considers this to be an indirect regenerative squamous metaplasia and that it is a reparative process which is secondary to the primary effect of direct destruction of the original epithelium by the hormone.

Gilten[17] believes that a true metaplasia of the uterine epithelium of rats following estrogen stimulation can occur. He suggests that through a forward extension into the uterus of the cervicoepithelium may occur, it is also possible that a stratified epithelium produced in the uterus by metaplasia may spread caudally to become continuous with the epithelium of the cervix.

From the data available in the literature on keratinizing metaplasia of the epithelium of the rat uterus, it is not clear whether avitaminosis

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TABLE I

Influence of Ovariectomy on Vitamin A Deficiency Induction of Epithelial Metaplasia in the Rat Uterus

Group	No. of Animals	Intact or Ovariecto-mized	Vitamin A	Presence of Squamous Meta-plasia
1	30	Intact	Deficient	Yes
2	10	Intact	Supplied	No
3	30	Ovariecto-mized	Deficient	No
4	10	Ovariecto-mized	Supplied	No

A exerts its influence directly on the epithelium independent of estrogen stimulation or whether it is dependent on estrogen for the induction of the keratinizing metaplasia. The experiments to be described in this paper were designed to provide information on the effect of ovariectomy on metaplastic changes in the uteri of vitamin A deficient rats, the manner of origin and development of stratified squamous epithelium in the uterus of the rat following estrogen stimulation and to provide information on the relationship of avitaminosis A to estrogen in producing keratinizing metaplasia of the epithelium of the rat uterus.

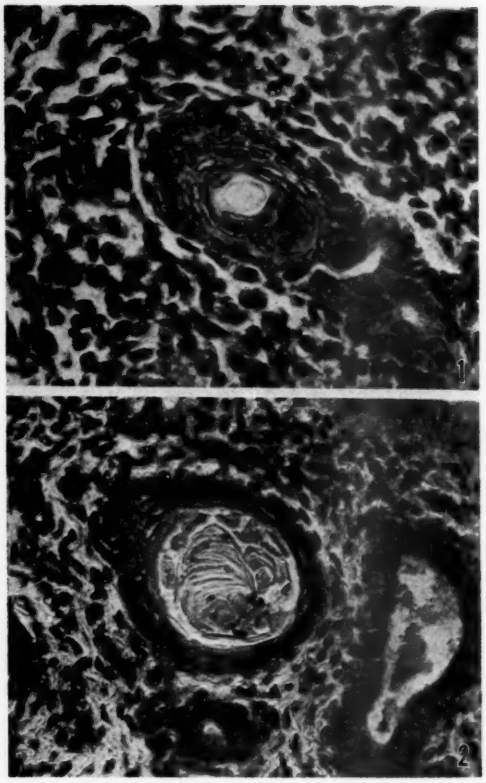
MATERIALS AND METHODS

Three separate experiments were carried out to study the problems mentioned above. Rats of the Wistar strain were used in all the experiments. The animals were killed with ether and the uteri fixed for twelve to twenty-four hours in Zenker's or Helly's solution. Serial sections were made of each uterus and every tenth section was mounted and stained with Weigert's hematoxylin and eosin. Specific information on the execution of the experiments along with the observations made will be presented under appropriate headings which follow.

Influence of Ovariectomy on Vitamin A Deficiency Induction of Epithelial Metaplasia[18]

Rats twenty to twenty-two days of age were divided into four groups. In Group 1 the animals were kept intact and placed on a vitamin A deficient diet.* The rats in Group 2 were treated as those in Group 1 except that they received a dietary supplement of vitamin A. The animals in Group 3 were bilaterally ovariectomized and placed on a vitamin A deficient diet. In Group 4 the rats were treated as in Group 3 except that they received a dietary supplement of vitamin A. Animals were autopsied from the eighth to the thirteenth week of the experiment.

In order to determine whether the daily food intake of the vitamin-deficient animals was an important factor in producing symptoms of avitaminosis A, rats of Groups 3 and 4 were pair-fed. The results are summarized in Table 1. Metaplasia of the uterine epithelium of the vitamin A deficient intact rats was similar to that previously reported (Figs. 1, 2 and 3).[1]



FIGS. 1 and 2. Photomicrograph of a portion of the endometrium of an intact vitamin A deficient rat demonstrating different degrees of squamous metaplasia of the glandular epithelium. The animal was autopsied during the twelfth week of the deficiency. The uterus was stained with Weigert's iron-hematoxylin and eosin. Original magnification ×100.

* Vitamin A Test Diet U.S.P. XIV, General Biochemicals, Inc., Chagrin Falls, Ohio.

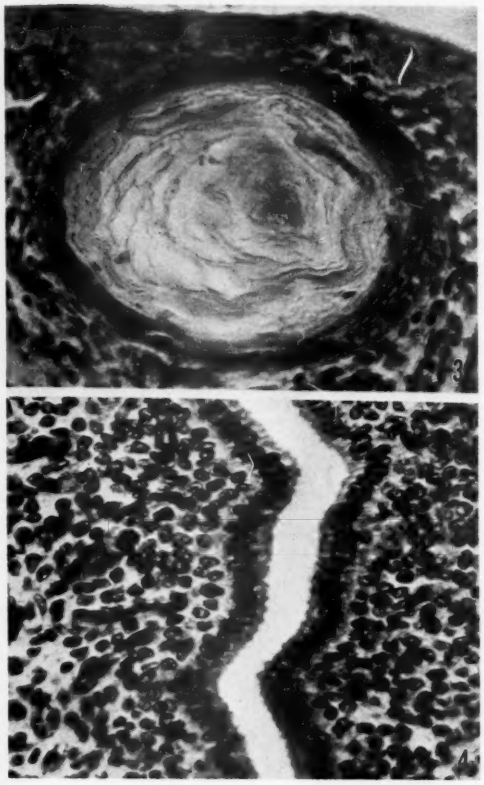


FIG. 3. Photomicrograph of a portion of the endometrium of an intact vitamin A deficient rat demonstrating extensive keratinization of the glandular epithelium. The animal was autopsied during the twelfth week of deficiency. The uterus was stained with Weigert's iron-hematoxylin and eosin. Original magnification ×100.

FIG. 4. Photomicrograph of a portion of the endometrium of an ovariectomized vitamin A deficient rat. Note the absence of any metaplastic changes. The animal was autopsied during the thirteenth week of deficiency. The uterus was stained with Weigert's iron-hematoxylin and eosin. Original magnification ×100.

However, no metaplasia of the uterus was observed in the vitamin A deficient rats following ovariectomy (Fig. 4). The results indicate that the ovaries also have an important role in producing metaplasia in the uteri of vitamin A deficient rats. Of the ovarian hormones, it is probably estrogen that is concerned with the metaplastic changes that occur in the uterine epithelium, since progesterone is primarily concerned with secretory activity of the epithelial cells whereas estrogen is primarily concerned with stimulating mitosis and cell growth.

TABLE II

Induction of Epithelial Metaplasia in the Rat Uterus by Weekly Administration of 2.0 mg. Estradiol Dipropionate Beginning at Seven Days of Age

No. of Animals	Days Autopsied after First Injection	No. of Animals Showing Metaplasia	Extent of Squamous Metaplasia
25	3–15	0	−
5	18	4	+
5	21	4	+++
5	24	4	+++
5	27	4	++
5	33	2	+++
5	39	4	++

Since the rats of Group 3 in the pair-fed series showed external manifestations of vitamin A deficiency and metaplasia of the lining epithelium of the trachea while the animals of Group 4 on the suppelemented diet, which were pair-fed, exhibited no manifestations of vitamin A deficiency, it could be concluded that the changes observed were due to avitaminosis A and not to indirect effects of the deficiency.

The Manner of Development of Squamous Metaplasia Induced by Estrogen[19]

Starting on the seventh day of age the rats were treated weekly with a single subcutaneous injection of 2 mg. estradiol dipropionate.* Animals were autopsied every three days from the third to the twenty-seventh day after receiving the first injection. Others were killed on the thirty-third and the thirty-ninth day after initial injection with estrogen.

The histologic structure of the infantile uterus of the seven day old rat was similar to that described by Wiesner (Fig. 5).[20] Squamous metaplasia was not observed in the uteri of the rats autopsied from the third through the fifteenth day after the first injection (Table II). However, the luminal columnar epithelial cells of the uteri of these animals were, for the most part, slightly hypertrophied, thereby indicating some response to estrogen stimulation had occurred. In a few of the

* The estradiol dipropionate used in this study was supplied through the courtesy of the Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

FIG. 5. Photomicrograph of a section of the uterus of animal autopsied on the seventh day of age, demonstrating the structure of the organ at the start of estrogen stimulation. Weigert's iron-hematoxylin and eosin. Original magnification ×160.

FIG. 6. Photomicrograph of a portion of the uterine epithelium showing early stage in the formation of the metaplastic epithelium. The intact original epithelial cells rest on the abnormal epithelium. The rat received a total dose of 8.0 mg. estradiol dipropionate. Weigert's iron-hematoxylin and eosin. Original magnification ×420.

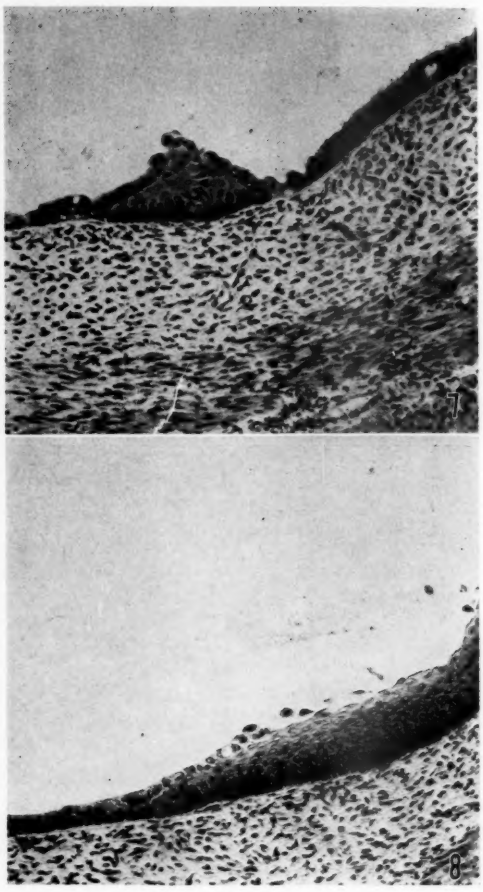


FIG. 7. Photomicrograph of a portion of the uterus, demonstrating further growth of the metaplastic epithelium. Original luminal cells resting on the stratified squamous epithelium. The animal received 8.0 mg. estradiol dipropionate. Weigert's iron-hematoxylin and eosin. Original magnification ×160.

FIG. 8. Photomicrograph of a portion of the uterus, demonstrating further extension of the metaplastic epithelium. The original luminal cells are sloughed off into the lumen. The animal received 10.0 mg. estradiol dipropionate. Weigert's iron-hematoxylin and eosin. Original magnification ×160.

animals the lining epithelium of the uteri consisted only of low columnar cells, while in other rats the luminal epithelium possessed a few vacuolated cells and appeared pesudostratified.

Squamous metaplasia of the uterine epithelium was found in the majority of the rats autopsied on the eighteenth through the thirty-ninth day after the first injection with estrogen. Metaplasia was observed to begin as separate foci of multiplication of the basal cells of the epithelium (Figs. 6 and 7). By this process the original columnar epithelial cells became separated from the basement membrane by areas of stratified epithelial cells (Fig. 8). With the loss of the original columnar cells and multiplication and differentiation of the new stratified epithelial cells numerous areas of the uterus came to be lined with strati-

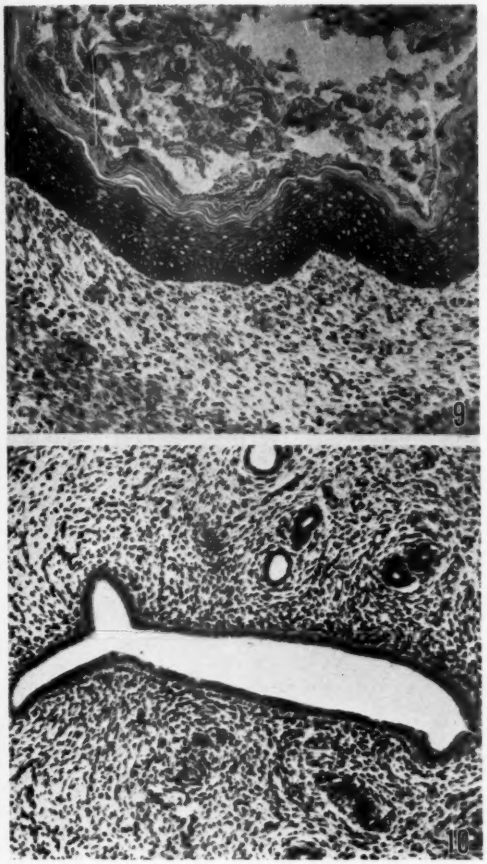


FIG. 9. Photomicrograph of a portion of the uterus, demonstrating extensive keratinization of the metaplastic epithelium. The dark-staining cells in the connective tissue of the endometrium are eosinophils. The rat received 8.0 mg. of estradiol dipropionate. Giemsa stain. Original magnification ×160.

FIG. 10. Photomicrograph of a portion of the uterus of an untreated intact rat showing the normal structure of the lining epithelium and of the glands. The animal was autopsied on the sixtieth day of age. Original magnification ×160.

fied squamous keratinizing epithelium (Fig. 9). With increase in multiplication and differentiation of the basal cells of the uterine epithelium the lumen was eventually lined with a continuous stratified squamous keratinizing epithelium. Normal epithelia is shown in Figure 10. Evidence that destruction of the original epithelium preceded formation of the stratified squamous epithelium could not be found in the material of this study.

Relation of Vitamin A Deficiency and Estrogen in Production of Epithelial Metaplasia[21]

Rats twenty to twenty-two days of age were divided into four groups. In Group 1 the animals were kept intact while the rats in Group 2 were bilaterally ovariectomized and all the animals were placed on the vitamin A free diet mentioned heretofore. In Group 3 the animals were bilaterally ovariectomized, maintained on a vitamin A deficient diet and treated with 1 μg. estrogen every third day. The animals in Group 4 were treated as those in Group 3 except that they received a dietary supplement of vitamin A. Animals in Groups 1 and 2 were autopsied from the seventh to the thirteenth week of the vitamin deficiency while rats of Groups 3 and 4 were autopsided from the fifth to the tenth week of the experiment.

The results are summarized in Table III. Uterine metaplasia was present in the uteri of intact vitamin A deficient rats while in the ovariectomized vitamin A deficient animals metaplasia of the uterine epithelium was not observed (Figs. 11–13). Keratinizing metaplasia occurred in the ovariectomized vitamin A deficient rats treated with estrogen (Figs. 14 and 15). The changes were observed to occur in the glandular epithelium before the lining epithelium. Uterine metaplasia was

TABLE III

Relation of Vitamin A Deficiency and Estrogen in Production of Epithelial Metaplasia in Uterus of the Rat

Group	No. of Animals	Intact or Ovariectomized	Vitamin A	Estrogen	Presence of Squamous Metaplasia
1	15	Intact	Deficient	No	Yes
2	15	Ovariectomized	Deficient	No	No
3	30	Ovariectomized	Deficient	Yes	Yes
4	30	Ovariectomized	Supplied	Yes	No

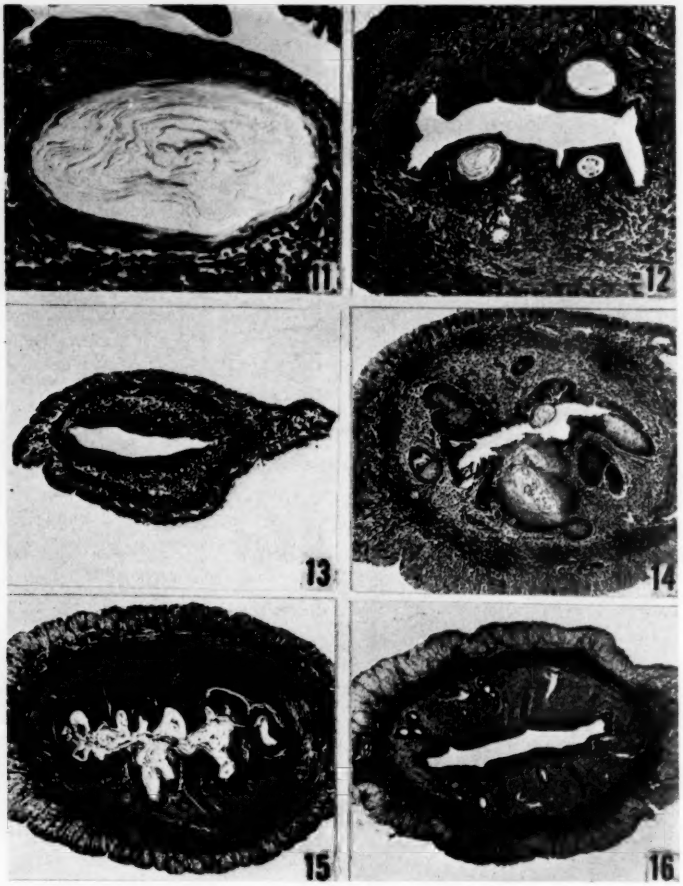


FIG. 11. Photomicrograph of a portion of the endometrium of an intact vitamin A deficient rat demonstrating extensive keratinization of the glandular epithelium. Keratohyline granules are distinctly shown. The animal was autopsied during the twelfth week of the deficiency. Weigert's iron-hematoxylin and eosin. Original magnification ×175.

FIG. 12. Photomicrograph of a portion of the uterus of an intact vitamin A deficient rat demonstrating stratification and keratinization of the glandular epithelium. The animal was autopsied during the twelfth week of the deficiency. Weigert's iron-hematoxylin and eosin. Original magnification ×70.

FIG. 13. Photomicrograph of the uterus of an ovariectomized vitamin A deficient rat. No evidence of uterine metaplasia is present. The animal was autopsied during the thirteenth week of the experiment. Weigert's iron-hematoxylin and eosin. Original magnification ×45.

FIG. 14. Photomicrograph of the uterus of an ovariectomized vitamin A deficient animal treated with estrogen. Many foci of uterine metaplasia are demonstrated. The rat was autopsied during the sixth week of the experiment. Weigert's iron-hematoxylin and eosin. Original magnification ×45.

FIG. 15. Photomicrograph of the uterus of an ovariectomized vitamin A deficient rat treated with estrogen. The entire lining epithelium is of the stratified squamous keratinized type. The rat was autopsied during the ninth week of the experiment. Weigert's iron-hematoxylin and eosin. Original magnification ×40.

FIG. 16. Photomicrograph of the uterus of an ovariectomized animal on an adequate diet and treated with estrogen. No evidence of uterine metaplasia. The animal was autopsied during the ninth week of the experiment. Weigert's iron-hematoxylin and eosin. Original magnification ×30.

not present in the ovariectomized animals on an adequate diet and also treated with estrogen (Fig. 16).

COMMENTS

It has been suggested by other investigators that squamous metaplasia of the uterine epithelium of rats treated with estrogen is not a direct effect of the hormone but a reparative process which is secondary to the primary effect of destruction of the epithelium by the estrogen.[15,16] The results obtained in the present investigation demonstrate rather clearly that uterine epithelial metaplasia, induced by estrogen, begins as many independent foci located along the uterine horns, which finally coalesce and replace the original epithelial cells with a stratified squamous keratinizing epithelium.

The phenomenon of metaplasia that occurs in epithelia is little understood. The cells that give rise to the stratified squamous epithelium have not been clearly identified. From his studies, Gilten[14] received the impression that, in some areas, a direct transformation of the columnar cells into stratified squamous epithelium occurred. Fluhmann[22,23] considers the transformation of the basal cells to a stratified squamous epithelium as indirect metaplasia. Motyloff[24] does not consider the presence of stratified squamous epithelium in the uterus as justification for assuming a process of metaplasia. According to Motyloff,[24] all incidences of ectopic stratified squamous epithelium could be explained by the heteroplastic development of the basal cells. From the present experiment it can be concluded that the basal cells give rise to the stratified squamous epithelium of the uterus following estrogen treatment.

In discussing the formation of stratified squamous epithelium in the uterus, the theory of Zuckerman[25] on the development of the epithelium has to be considered. This theory maintains that the formation of stratified squamous epithelium due to estrogen treatment is the primary response of tissue in whose development the urogenital sinus played a direct or an indirect part. It is unlikely that the observations from the present investigation support the theory, for multiple foci of stratified squamous epithelium were located throughout the uterine horns. In order to explain the foci of metaplastic epithelium, according to Zuckerman's theory, it would be necessary to postulate the migration of cells from the urogenital sinus. Fluhmann[22,23] considers the basal cells to arise from division of the columnar cells.

Destruction of the original epithelial cells was not observed to precede the development of the metaplastic epithelium. The change appears to be a metaplasia resulting from the direct action of estrogen on the basal cells and not an indirect regenerative squamous metaplasia.

The epithelial metaplasia produced by estrogen stimulation differs from the metaplasia which occurs in vitamin A deficiency in that, in the estrogen-induced metaplasia, the change takes place in the luminal epithelium, while in avitaminosis A the changes take place in the endometrial glands. The epithelial metaplasia in the rat uterus produced by estrogen stimulation was more pronounced than that produced by avitaminosis A.

It has been suggested by other investigators that squamous keratinizing metaplasia of the uterus results from a local vitamin A deficiency regardless of any other factors.[26] The results obtained from the present investigations do not support that conclusion. If all keratinizing metaplasia is due to a local vitamin A deficiency regardless of any other factors, then such changes would be present in the uteri of ovariectomized vitamin A deficient rats. The observations made in the present studies demonstrate rather clearly that uterine metaplasia following avitaminosis A occurs only when the epithelial cells are under the influence of estrogen. The synergistic action of vitamin A deficiency and estrogen in producing uterine metaplasia is evident by the pronounced metaplastic changes observed in the ovariectomized rats on a vitamin A deficient diet and treated with estrogen, as compared to the absence of changes in the ovariectomized animals on a vitamin A free diet, or in the ovariectomized rats on an adequate diet treated with estrogen.

No definite relationship exists between vitamin A deficiency and estrogen in producing abnormal cornification of the vagina, the change occurs in the intact as well as in the ovariectomized vitamin A deficient rats.[27–29] It has been reported that cornification of the

vagina following estrogen treatment is altered by excess vitamin A.[30-31] Recently, tissue culture technics have been used to study this relationship between vitamin A and estrogen in producing keratinized epithelium. The vagina has primarily been used for this study. It has been demonstrated that estrogen can produce cornification of the mouse vaginal epithelium *in vitro*.[32] Also, *in vitro*, keratinization of the rat vagina was observed to take place in cultures that contained a standard medium.[33] The keratinization that occurred was probably due to a lack of available vitamin A. The uterus, up to the present time, has not been used for *in vitro* studies. However, if such studies were undertaken, interesting observations would be made on this problem of the relationship between vitamin A deficiency and estrogen in producing keratinizing metaplasia in the uterus of the rat.

These observations indicate that the abnormal growth that occurs in the uterus and that which has beer reported to occur in the vagina following avitaminosis A cannot be considered to be the same. Although the end result, a keratinized epithelium, is the same, the cytologic processes involved in producing the change are different in the two organs. In the vagina the abnormal growth is more of a hyperplastic change and, as just mentioned, occurs in the intact and ovariectomized rat, while in the uterus the growth is a true metaplasia and occurs only in the intact animal. This difference in response of the uterus and vagina to vitamin A deficiency may result from the difference in the embryology, morphology and physiology of the two organs. The conclusions drawn from observations made on the vagina in avitaminosis A, or between vitamin A and estrogen, cannot be generalized to include the uterus.

The question that arises in regard to keratinized epithelium is which of the two causes, estrogen or vitamin A deficiency, is the primary factor in producing the change. On the basis of the present work it can be concluded that vitamin A deficiency *per se* cannot be considered to be the primary factor in inducing keratinizing metaplasia in the uterus of the rat. Estrogen seems to have an equal role in producing the change. On the other hand, from the experimental data that are available on the rat vagina, vitamin A deficiency seems to be the primary factor in producing abnormal cornification.

Although vitamin A suppresses the cornification of the vagina induced by estrogen, it cannot be definitely concluded that vitamin A plays a general role in the prevention of all keratin formation. In the epidermis of the male rat, topically applied vitamin A dissolved in sesame oil did not interfere with the keratinization process.[34] Likewise, in the guinea pig, vitamin A dissolved in oleic acid, linoleic acid or alcohol did not inhibit the formation of the stratum corneum of the skin.[35] Also, vitamin A did not prevent the estrogen-induced hyperkeratosis of the nipple epidermis in the guinea pig.[34]

The mode of action of vitamin A in maintaining normal uterine epithelium is not clear. Since vitamin A has not been demonstrated by fluorescence microscopy in epithelia that are known to undergo keratinization in depleted animals, it has been suggested that vitamin A *per se* is not responsible for maintaining normal epithelia but a metabolite of vitamin A may be involved.[36] It seems that in order to maintain the integrity of the uterine epithelia a balance exists between vitamin A and estrogen and when this balance is disrupted a keratinizing metaplasia occurs.

SUMMARY

Metaplasia of the uterine epithelium of estrogen-treated rats begins as many independent foci that grow and coalesce to produce a keratinized stratified squamous epithelium which replaced the original uterine epithelium. The origin of epithelial metaplasia produced by estrogen stimulation differs from the metaplasia which occurs in vitamin A deficiency in that in the former the change takes place in the luminal epithelium and the latter in the endometrial glands. The epithelium produced by estrogen stimulation is thicker and more heavily keratinized than that produced by vitamin A deficiency.

Keratinizing metaplasia in the uterus of the rat was observed to occur only in intact animals on a vitamin A deficient diet or in the ovariectomized, vitamin A deficient rats treated with estrogen. The results demonstrated that estrogen has an important role in producing keratinized epithelium in avitaminosis A and the

change cannot be considered to be due to only a local vitamin A deficiency.

From the present investigation it can be concluded that vitamin A deficiency is not the primary factor in producing keratinizing metaplasia in the uterus of the rat since estrogen plays a role in producing the change.

The mode of action of vitamin A in maintaining normal uterine epithelium is not known, but it seems that a balance may exist between estrogen and vitamin A in order to maintain the integrity of the uterine epithelium. When this balance is disrupted, keratinizing metaplasia occurs.

The abnormal cornification of the vagina that occurs in vitamin A deficiency of the rat was compared with the uterine changes and it was concluded that the two are not the same. In the vagina the change is more of a hyperplastic change while in the uterus it is a metaplastic change. Therefore, the conclusions drawn from the observations made on the vagina in avitaminosis A and between vitamin A and estrogen cannot be generalized to include the uterus.

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The Biologic Activity of Vitamin A Acid

JOHN E. DOWLING, PH.D.

VITAMIN A ACID was first tested biologically by Arens and Van Dorp,[1] who isolated crystalline vitamin A acid as an intermediate in their synthesis of vitamin A. On feeding vitamin A acid to deficient rats, they found that the animals promptly began to grow again and were relieved of their deficiency symptoms. Arens and Van Dorp first assumed that this activity was the result of reduction of the acid in the body to vitamin A alcohol. To check this assumption, they gave massive doses of vitamin A acid to depleted animals and extracted their tissues for vitamin A. Regardless of the amount of acid administered, however, no vitamin A alcohol could be detected in the livers of these animals.[2] Consequently, they suggested that perhaps the acid itself is biologically active and is not reduced *in vivo*.

Shortly thereafter, Sharman[3] confirmed that vitamin A acid has intense growth-promoting power. Sharman likewise was unable to detect any vitamin A alcohol in the tissues of acid-dosed rats, and reported also that he was unable to detect any vitamin A acid in the tissues. Sharman agreed with Arens and Van Dorp that vitamin A acid probably exerts its activity without being reduced to either vitamin A alcohol or aldehyde.

Moore,[4] commenting on these findings several years later, pointed out that if vitamin A acid exerts its activity without being reduced *in vivo*, then it is unlikely that the acid can replace vitamin A in the visual cycle, which demands the alcohol or aldehyde as precursor of the visual pigments.[5]

We have recently shown that Moore's hypothesis is correct.[6] Young weanling rats raised on a vitamin A deficient diet supplemented with vitamin A acid become highly nightblind, and eventually completely blind, though continuing to grow normally and to remain otherwise in excellent condition.

Figure 1 summarizes the results of one such experiment. Two weanling animals were raised on the vitamin A deficient diet, but one animal was supplemented with vitamin A acid from the start of the experiment. Both animals grew at about the same rate for five to six weeks. Then the unsupplemented animal stopped growing, rapidly lost weight, and died on the fifty-seventh day of the experiment. The other animal continued to grow and appeared to remain in excellent condition, as the photograph taken on the one-hundred-fifty-seventh day of the diet is intended to show.

On the same day the rat's electroretinogram (ERG) was recorded. This is shown at the right of the figure, compared with that of a normal animal measured at the same time. The vitamin A acid animal, though normal in weight and appearance, was highly nightblind. Its visual threshold (the luminance of a 1/50 second flash needed to excite a just measurable ERG) was raised about 3.25 log units (about 1,800 times) above normal. Rhodopsin extracted from the retinas of animals in this condition is present in only 1 to 3 per cent of normal amounts.

Animals raised on a vitamin A deficient diet supplemented with vitamin A acid grow as fast and as well as animals on the deficient diet supplemented with vitamin A alcohol. We have compared the growth and appearance of groups of vitamin A acid- and alcohol-supplemented animals for periods of five to six months, and find them similar in all respects.[6] The gross appearance of the internal organs on autopsy, and the histologic appearance of the tracheal epithelium is the same in both groups.

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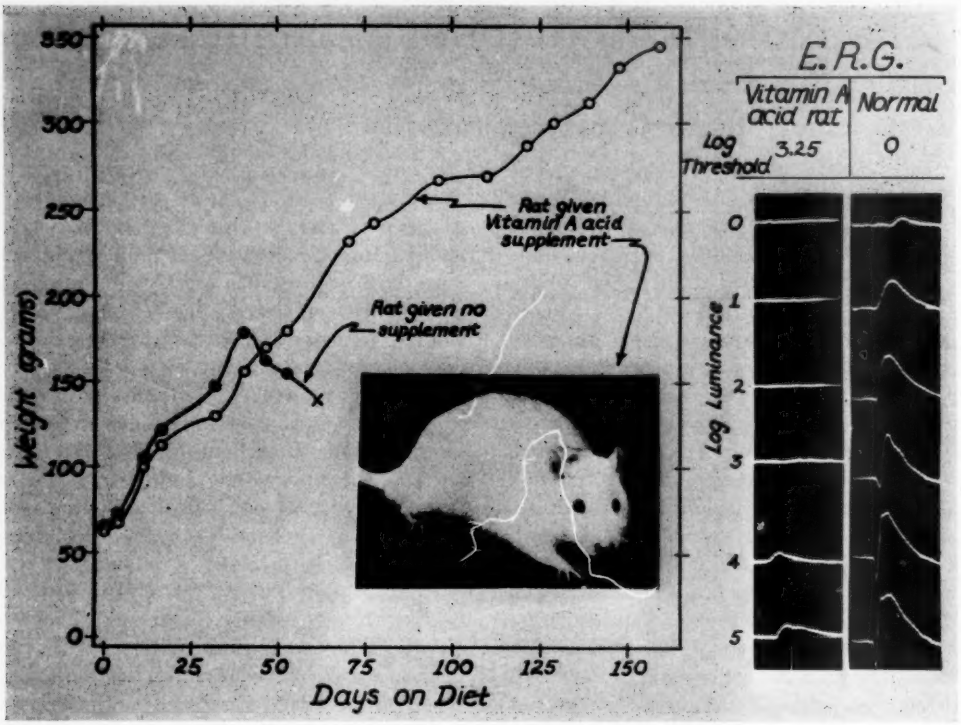

FIG. 1. Activity of vitamin A acid. Weanling rats were placed on a vitamin A-deficient diet, but one was supplemented with vitamin A acid. The rat given no supplement died after fifty-seven days on the diet; the animal receiving vitamin A acid continued to grow and remained in excellent condition for the duration of the experiment, a little over five months. The picture of this animal was taken at the end of the experiment, as were the electroretinograms shown at the right, compared with those of a normal animal. They show this rat to be highly nightblind: its visual threshold had risen 3.25 log units (1,800 times) above normal, and only just detectable ERG's could be evoked even at the highest luminances.

At the time of writing this report, we have observed one rat on this regimen for eighteen months, and it still appears perfectly healthy.

For our experiments, the supplements of vitamin A acid are dissolved in vegetable oil and given orally three times a week by means of a syringe fitted with a blunted needle. In most experiments, we feed at a dosage level of 50 μg. of vitamin A acid per day. This high level was chosen with the thought that if the acid possesses the lowest activity yet reported, about 10 per cent of vitamin A, we would still provide the equivalent of at least twice the minimal daily demand for vitamin A.

In recent experiments we* have raised groups of animals on vitamin A-deficient diets supplemented with 0.5, 5, 50, and 500 μg. of vitamin A acid per day. The supplements were first administered a few days after the animals had begun to level off in weight. At a dosage level of 0.5 μg. per day, little if any vitamin A activity is noted: the animals have all died within a few days after control animals fed no supplements or fed vegetable oil alone. The animals fed supplements of vitamin A acid at dosage levels of 5 or 50 μg. per day, however, quickly resume growth, continue to grow at the normal rate, and assume a normal appearance.

Rats fed at the dosage level of 500 μg. per day also begin growing again immediately, but continue to grow for only one to three weeks. They then level off in weight, begin to show signs of hypervitaminosis A (such as

* I should like to thank Mr. David Jewett for assisting with these experiments.

bleeding about the nose and eyes, loss of hair, etc.), and after five to six weeks all have died. This result confirms the report of Thompson and Pitt that high doses of vitamin A acid readily produce signs of hypervitaminosis A.[7] Thompson and Pitt have also shown that vitamin A acid causes symptoms of hypervitaminosis A at considerably lower dosage levels than does vitamin A itself.

On extracting the tissues of animals fed various levels of vitamin A acid, we have confirmed Arens and Van Dorp, and Sharman, in failing to detect any vitamin A or vitamin A acid in liver, blood, or kidney.[6] Redfearn[8] has also recently made similar observations, but finds that after large doses of the methyl ester of vitamin A acid, one can detect small amounts of this substance in the body fat, though none in the liver or kidney.

One consequence of the failure of animals to store vitamin A acid is that the animals can not long withstand interruption of the supplements. Within a week the animals begin to level in weight and to show signs of the deficiency. If supplements are not resumed, the animals lose weight rapidly and die within three to four weeks.[6] Redfearn has confirmed this observation, but reports that rats fed the vitamin A acid methyl ester tolerate the interruption of this supplement for longer periods of time.[8]

Supplements of vitamin A acid also do not conserve stores of vitamin A alcohol already present in the liver. We had noted that animals supplemented with vitamin A acid from a few days after weaning become nightblind as rapidly as animals given no supplements.[6] During the first few weeks on a deficient diet, animals deplete their livers of vitamin A; and it appeared from the preceding observation that this depletion might occur at the same rate whether vitamin A acid was administered or not. We confirmed this suspicion by measuring directly the disappearance of vitamin A from the liver. It turned out that the vitamin A content of the liver declines at the same rate in unsupplemented and vitamin A acid-supplemented groups of animals.[6]

As already said, the rise of visual threshold, nightblindness, occurs as promptly and develops at the same rate in animals supplemented with vitamin A acid as in animals receiving no supplements. After sixty days or so, however, animals receiving no supplements have all died, while animals receiving vitamin A acid remain well but continue to become more nightblind. Eventually (in eight to ten months), these animals become completely blind. At this time we no longer can record any electrical responses from the eyes, even with stimuli 7 log units (about 10 million times) above the threshold level. We can also no longer detect any rhodopsin in these retinas.[6]

The structure of the visual cells in the retinas of these animals also gradually degenerates as the night blindness progresses.[9] The degeneration begins in the outer segments of the rods (after two months or so) but eventually includes the rest of the visual cell, so that, after ten months, the visual cells have completely disappeared except for a few residual nuclei, pressed between the bipolar cells of the retina and the pigment epithelium. The rest of the retina (ganglion and bipolar cells, and pigment epithelium) remain normal in appearance; only the visual cells are affected in this condition.

If vitamin A is given to animals supplemented with vitamin A acid, visual recovery occurs in varying degree, depending on the extent of retinal degeneration. Visual cells which have not deteriorated too greatly can regenerate outer segments, but entire visual cells which have been lost are not replaced.[9]

It appears from these experiments that the only role for which specifically vitamin A is required in the rat is as precursor of the visual pigments. For all other, systemic functions of the vitamin, vitamin A acid seems to serve equally well. It has been suggested that vitamin A acid may either be the active principle for the tissue functions of vitamin A or an intermediate between vitamin A and the active principle.[4,6] The metabolism of vitamin A can be expressed in such a diagram as shown in Figure 2.

Vitamin A is stored in the tissues as vitamin A ester and is transported as the free alcohol. It may be reversibly oxidized to vitamin A aldehyde (retinene) to form the visual pigments. It appears to be irreversibly oxidized to vitamin A acid, which may either serve as

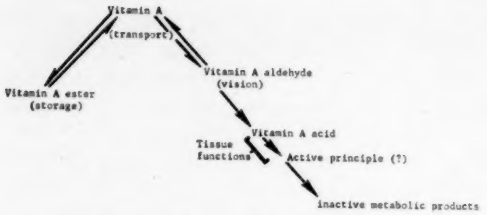

FIG. 2. General metabolism of vitamin A.

the active principle or may be converted to the active principle.

The failure to detect vitamin A acid in the tissues of rats dosed with this substance may favor the latter hypothesis and has prompted us to test possible oxidation products of vitamin A acid for growth-promoting potency. We* have now tested the C_{19} acid, the C_{18} ketone, the C_{17} acid and the C_{14} aldehyde, but have not found any of these compounds to display vitamin A activity.

SUMMARY

The literature concerning the activity and metabolism of vitamin A acid has been reviewed. Recent experiments testing the potency of vitamin A acid at various dosage levels are reported, along with experiments testing the vitamin A activity of several possible oxidation products of vitamin A acid.

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* I should like to thank Mr. David Jewett for assisting with these experiments.

DISCUSSION

DR. G. J. WRIGHT (*Urbana, Illinois*): When the work of Drs. Dowling and Wald on vitamin A acid became known, we naturally were interested to see if we could detect the presence of this acid in any of our systems. Using the technic of carrier crystallization, we attempted to detect the formation of radioactive vitamin A acid from C^{14}-labeled vitamin A acetate in normal pig adrenal homogenates and in tissues of rats which had been injected with the C^{14}-labeled material. The results of these experiments are shown in Tables I and II. As can be seen from these data, no vitamin A acid could be detected. It seems reasonable to assume that if vitamin A acid were the "active" form of the vitamin its presence could be detected by these means. These experiments, of course, do not preclude the possibility that vitamin A acid is an intermediate with an extremely rapid turnover rate.

TABLE I

Nonformation of Vitamin A Acid by Adrenal Homogenates*

Crystallization	Recovered Carrier (mg.)	Specific Activity (c.p.m./mg.)	Total Activity (c.p.m.)
...	5	...	2,560
1	3.9	104	406
2	2.2	220	44
3	1.2	0	...

* Each incubation included 3 ml. of homogenate, adenosine triphosphate, 2×10^{-3} mM., TPNH, 1.4×10^{-3} mM., vitamin A acid, 15 μg., 0.05 μc. vitamin A-1′, 9-C^{14} acetate (7 μg.).

TABLE II

Vitamin A Acid Carrier Crystallization with Water-soluble "Metabolite" of C^{14}-Vitamin A Acetate from Rat Liver

Crystallization	Recovered Carrier (mg.)	Specific Activity (c.p.m./mg.)	Total Activity (c.p.m.)
...	43.4	...	13,300
1	40.6	76	3,085
2	23.5	14	329
3	12.3	0	...

Vitamin A Function in Ubiquinone and Cholesterol Biosynthesis

OSWALD WISS, M.D.,* URS GLOOR, PH.D. AND FRITZ WEBER, PH.D.

PREVIOUS WORK provides little evidence linking vitamin A with cholesterol metabolism. Although Ralli and Waterhouse[1] found a higher blood level of cholesterol in vitamin A deficiency, this observation was not confirmed by Green et al.,[2] who found that the cholesterol content of blood and liver was unchanged in vitamin A deficiency. No definite influence of vitamin A deficiency on the incorporation of labeled acetate into the cholesterol molecule could be detected by Wolf et al.[3]

A distinct relationship between vitamin A deficiency and ubiquinones was observed by Morton and his group[4–7] in 1953. Studies on the alteration of the unsaponifiable fraction in the liver of the vitamin A deficient rat gave the first hint of the existence of a new class of substances, the ubiquinones. Vitamin A deficiency is associated with an increase in substances characterized by distinct ultraviolet spectra; one of these, called substance SA, was found to be ubiquinone (Fig. 1).

After the structure of the ubiquinones had been elucidated and proved[4] to be identical coenzyme Q, detected by Lester et al.,[8] we became interested in its biosynthesis and in the functional relationship between ubiquinone and vitamin A deficiency.

In a first experiment carried out in 1958,[9] we found that 2-^{14}C-labeled mevalonic acid is incorporated into the ubiquinone molecules of rat liver and that the specific activity of the isolated ubiquinone is about the same as that of the isolated cholesterol. Considering the polyisoprenoid structure of the side-chain, it did not come as a surprise to find that mevalonic acid is its precursor. As regards the structural relationship between ubiquinone and the E and K vitamins, where a polyisoprene side-chain is always attached to an aromatic or a quinone derivative (Fig. 2), it was somewhat unexpected that such a synthetic reaction takes place in the animal body. On the other hand, good although indirect evidence that a vitamin K_2 form is synthesized in the rat using methylnaphthoquinone as a precursor, had already been provided by Martius and Esser.[10]

To give an exact proof for this biosynthesis reaction and to determine which part of the molecule was labeled—whether the whole side-chain and nothing but the side-chain is synthesized by mevalonic acid—it seemed to us necessary to degrade the biosynthetically labeled ubiquinone chemically and to localize the radioactivity in the molecule. Before doing so, however, another problem had to be cleared up.

In our first experiment on the incorporation of labeled mevalonic acid into the ubiquinone, chromatography on alumina was used for its isolation. Later on it turned out that rat liver contains a mixture of different isoprenologues of the ubiquinones, the main compound being the ubiquinone (45) with about 80 per cent, ubiquinone (50) with about 20 per cent, and very small amounts of lower isoprenologues in the range of 1 per cent. These results have been achieved by Linn et al.,[11] Lawson et al.,[12] Olson and Dialameh[13] and ourselves.[14]

It was therefore necessary to determine whether mevalonic acid is incorporated into both main ubiquinones of the rat liver. After parenteral administration of labeled mevalonic acid (250 μg., or 4 μC. to each rat), the mixture of ubiquinone(45) and (50) was isolated and the components separated by paper chroma-

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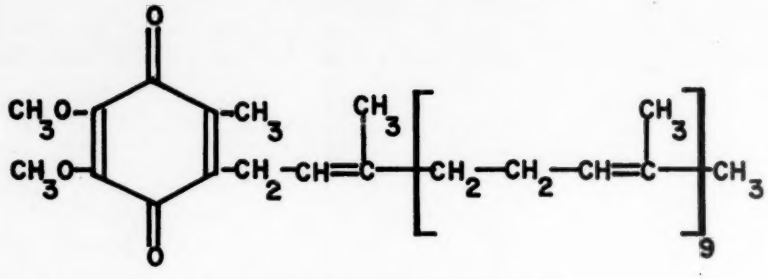

FIG. 1. Ubiquinone (50) = coenzyme Q_{10}.

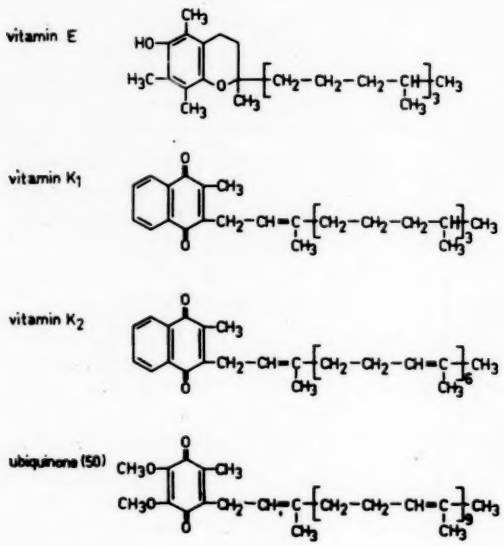

FIG. 2. Structural relations between vitamins E, K_1, K_2 and the ubiquinones.

tography. A clear separation was achieved. It turned out that both ubiquinones were active and that the activity corresponded to the amount of the isolated isoprenologues.[15] According to these results we did not consider it necessary to separate the mixture of ubiquinone(45) and (50) in our degradation experiments.

On the other hand, in order to obtain clear information on the biosynthesis of the ubiquinone side-chain, incorporation experiments with two differently labeled mevalonic acids were necessary (Fig. 3). The 2-^{14}C-mevalonic acid was synthesized in our laboratories by Dr. Würsch, the 4-^{14}C-mevalonic acid was kindly supplied by Dr. Cornforth of the National Institute for Medical Research in London. In two corresponding experiments each of the two mevalonic acids were given orally to rats (50 μg. of 2-^{14}C-mevalonic acid, 4 μC./animal, and 1.5 mg. of 4-^{14}C-mevalonic acid, 0.5 μC./animal totally).

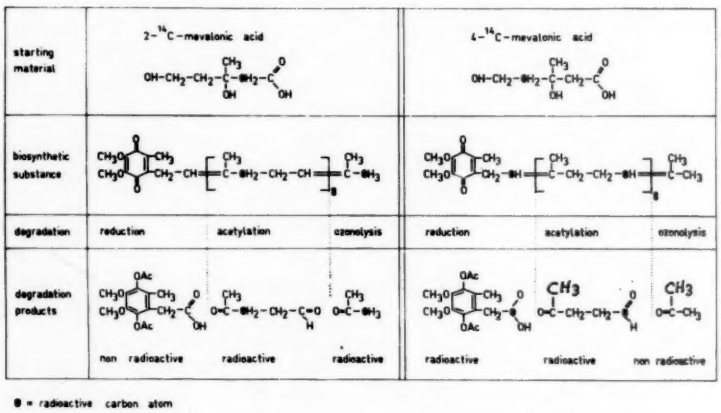

FIG. 3. Biosynthesis and degradation of ubiquinone from two differently labeled mevalonic acids.

TABLE I

Radioactivity of the Degradation Products of Biosynthetically Labeled Ubiquinones after Dosing 2-^{14}C-Mevalonic Acid and 4-^{14}C-Mevalonic Acid

Degradation Product	Starting Material for Biosynthesis	
	2-^{14}C-Mevalonic Acid	4-^{14}C-Mevalonic Acid
Material degraded by ozonolysis	104	4.9
Substituted phenylacetic acid	0	4.5
Laevulinaldehyde (derivative)	114	3.2
Acetone (derivative)	108	0.15

Figures = c.p.m./μM. (liquid scintillation counting).

The biosynthetically labeled ubiquinones of the liver were obtained in crystalline form. After reduction, acetylation and ozonolysis, the substituted phenylacetic acid and the derivatives of laevulinaldehyde and of acetone were isolated and their radioactivity measured.

Assuming that the whole side-chain, but only the side-chain, is synthesized by mevalonic acid according to the pattern of steroid synthesis and that all isoprene units are connected asymmetrically, the radioactivity should be distributed in the different degradation products as indicated in Figure 3. From 2-^{14}C-mevalonic acid labeled acetone and labeled laevulinaldehyde, only the substituted phenylacetic acid should be obtained unlabeled. The acetone derived from 4-^{14}C-mevalonic acid should contain no radioactivity, whereas laevulinaldehyde and the carboxy group of the substituted phenylacetic acid should be active.

Table I shows that the radioactivity distribution was found as expected.

After 2-^{14}C-mevalonic acid no radioactivity in the substituted phenylacetic acid was found. The activity in the laevulinaldehyde and acetone derivative corresponds quite well with the theoretic value: 114 and 108 counts instead of 104. According to the lower activity of the 4-^{14}C-mevalonic acid the isolated degradation products were also less active. Also in this experiment a rather good correlation with the theoretic value was obtained: practically no activity in the acetone molecule; the activity in the two other degradation products was within the expected range. In our first experiment[16] dinitrophenylhydrazine was used for isolating both acetone and laevulinaldehyde. Because of the quenching effect by scintillation counting, we now preferred the semicarbazone of the acetone. Laevulinaldehyde was transformed into phenylmethyldihydropyridazine by addition of phenylhydrazine.[17]

Rudney[18] found a good incorporation by yeast of labeled formate in the methoxy group of ubiquinone. However, only a very small incorporation of labeled formate into rat liver ubiquinone could be detected. Being aware that formate may enter the acetate pool and thus be incorporated into the side-chain of ubiquinone, we compared the incorporation ratio of formate into rat liver ubiquinone, rat liver cholesterol and rat liver fatty acid (Table II).

After administration of formate, as indicated in Table II, the incorporation ratio in the different substances is in the same range, very

TABLE II

Biosynthesis of Ubiquinone in Rat Liver Starting from Formate or Methionine

Substance Administered	Total Amount of Radioactivity Administered	Specific Radioactivity (Disintegration/minute [Absolute] per μM.)			
		Squalene	Ubiquinone	Cholesterol	Palmitic Acid Methylester
Sodium formate*	1 mc.	...	420	297	163
Methionine†	0.5 mc.	7	5,900	13	3

NOTE: The animals were decapitated two hours after dosing.

* Sodium-formate-^{14}C; 90 μC./mg., ten rats per group (100 gm. animals).

† Methionine (methyl-^{14}C); 16 μC./mg., administration per os in 0.3 ml. saline.

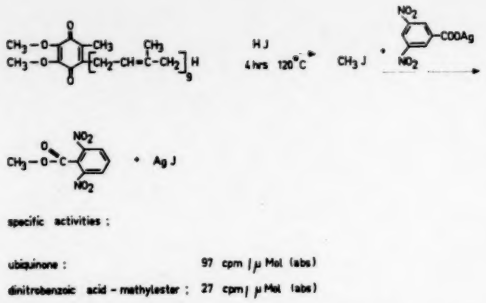

FIG. 4. Degradation of ubiquinone (45) isolated after dosing rats with methionine (methyl^{14}-labeled).

low, and must be attributed to acetate formed by formate. The somewhat higher specific activity of the ubiquinones does not indicate an additional incorporation of formate in the methoxy group, because there is about the same difference in specific activity between cholesterol and fatty acid. These results may be explained for instance by a dilution effect of exogenous cholesterol and fatty acid. Conclusive results, however, were obtained by administration of methyl-labeled methionine. We found a high activity in the ubiquinones and practically no incorporation in the cholesterol. Hence it becomes probable that a methoxy group is formed by transmethylation. To obtain conclusive proof, we carried out splitting of ether, using 4 mg. of labeled isolated ubiquinone together with 300 mg. of inactive ubiquinone(45). The methyliodide formed was transformed to dinitrobenzoic acid ester and isolated. Figure 4 shows that it was found to be active. Dinitrobenzoic ester, containing only one methyl group, can theoretically be half as active as the ubiquinone on a molar basis. Therefore, only about 50 per cent of the theoretic value was obtained; in a second experiment about 65 per cent was found. It can, therefore, not be excluded that the methyl group in 5-position of the benzoquinone ring is also derived from methionine. The difference in formate incorporation between yeast and rats, as found by Rudney,[18] is explained by the fact that the labile methyl of methionine can be synthesized by yeast and is practically essential for higher animals.

No conclusive results have been obtained up to now on the precursor of the benzoquinone ring system. Using random labeled phenylalanine, Olson et al.[19] found an incorporation into the ubiquinone(45) of rat liver. In addition to ubiquinone, however, cholesterol was also found to be labeled, the specific activity of the ubiquinones being somewhat higher. This may be an indication that in addition to the acetate formed by degradation of phenylalanine the ring component is used as such for the benzoquinone part of the molecule. Table III shows our results obtained after administration of random labeled tyrosine. As expected, ubiquinone and cholesterol were found to be active. In addition a smaller activity in the squalene and fatty acid could be seen. In our experiment the specific activity of cholesterol was found to be higher than that of ubiquinone. The ratio is similar to that after mevalonic acid administration. Considering these results, we think it unlikely that tyrosine is metabolized to a precursor of the ring component of the ubiquinones. We believe that, were this the case, a considerably higher specific activity of the ubiquinones than of cholesterol should be observed. Degradation experiments with labeled ubiquinone are under way. Thus we hope that this question can soon be answered.

As mentioned before, the first indication for a relationship between ubiquinone and vitamin A was given by Morton and his group,[4] who showed that the so-called substances SA and SC were markedly increased in vitamin A deficiency. In this early stage of the investigations it was supposed that SA and SC might be metabolically related to cholesterol in that they are metabolic intermediates which accumulate in the vitamin A deficiency state.[20] After it had turned out that ubiquinones are not steroids, but that mevalonic acid is a precursor for ubiquinone, it appeared of interest to prove whether there is actually a relationship between the ubiquinone and cholesterol synthesis.

In normal rats the total incorporation ratio into the unsaponifiable matter of liver is rather independent of the interval between administration and sacrificing of the animals, whether mevalonic acid is given orally or parenterally (Table IV).

Table V shows that the incorporation corresponds to the doses given, further the normal range of the incorporation of 2-^{14}C-mevalonic

TABLE III

Biosynthesis of Ubiquinone in Rat Liver Starting from Tyrosine and Mevalonic Acid

Substance Administered	Total Amount of Radioactivity Administered	Specific Radioactivity (Disintegration/minute [Absolute] per μM.)			
		Squalene	Ubiquinone	Cholesterol	Palmitic Acid Methylester
Tyrosine*	500 μC.	317	3,270	6,200	300
Mevalonic acid†	25 μC.	11,250	6,260	18,120	140

Note: The animals were decapitated two hours after dosing.
* Tyrosine (uniformly labeled); 46 μC./mg., ten rats per group (100 gm. animals).
† 2-^{14}C-mevalonic acid; 16 μC./mg., administration per os in 0.2 ml. saline.

acid in the unsaponifiable matter and into cholesterol, ubiquinone and squalene of rat liver.

Table VI shows differences of the mevalonic acid incorporation between a normal, pair-fed rat and another affected by severe vitamin A deficiency.

The method used was the same as in the experiments mentioned previously. The separation was achieved by chromatography on alumina, inactivated by addition of 7 per cent water. About fifty fractions were collected with a fraction collector, and the radioactivity measured in each sample. No great difference was observed in either normal or vitamin A deficient animals between the total incorporation into the unsaponifiable matter. The distribution of the radioactivity between

TABLE IV

Incorporation of 2-^{14}C-Mevalonic Acid into Unsaponifiable Matter of Rat Liver after Oral and Intraperitoneal Administration

Administration	Hours Between Administration and Sacrificing	Radioactivity in the Unsaponifiable Matter	
		Counts/Minute	Per Cent of Dose
Intraperitoneal	0.5	120,300	8.5
	1	114,800	8.1
	2	81,100	5.8
	4	77,800	5.5
	8	43,000	3.3
Oral	0.5	135,000	9.5
	1	160,300	11.3
	2	149,000	10.5
	4	133,600	9.4
	8	60,400	4.3

NOTE: Rats of 80 to 100 gm. 50 μg. mevalonic acid in 0.2 ml. saline (representing 1.4×10^6 counts/minute).

TABLE V

Incorporation of Increasing Doses of 2-^{14}C-Mevalonic Acid into Unsaponifiable Matter

Mevalonic Acid (mg.)	Radioactivity in Unsaponifiable (per cent of dose)	Total Unsaponifiable = 100 Per Cent Radioactivity		
		Squalene	Ubiquinone	Cholesterol
0.25	8.0	2.1	1.4	96
0.5	7.0	2.6	2.2	95
1	9.9	1.5	2.1	96
2	6.0	3.6	3.9	91
4	9.2	0.3	6.6	89
8	8.6	0.6	0.7	98
16	8.5	1.8	2.7	91
32	5.5	2.1	0.9	97

NOTE: Rats of about 50 gm. each dose of mevalonic acid in 0.3 ml. saline. Interval between administration and sacrificing was two hours. Liquid scintillation counting.

TABLE VI

Differences of Mevalonic Acid Incorporation

Mevalonic Acid	Normal Rat (%)	Vitamin A Deficient Rat (%)
Cholesterol	95	47
Ubiquinone	2.5	14
Squalene	2	39
Total Incorporation	98,250 c.p.m.	77,700 c.p.m.

TABLE VII

Incorporation of 2-^{14}C-Mevalonic Acid into Squalene, Cholesterol and Ubiquinone (Liver Homogenates)*

Liver Homogenates	10.6×10^6 c.p.m. of 2-^{14}C-Mevalonic Acid Added (incorporated radioactivity in c.p.m.)		
	Incubated Under O_2		In Air
Squalene.....	52,700	44,300	176,900
Cholesterol....	807,300	862,100	539,700
Ubiquinone....	<300	<650	<130

* Test conditions: 3 ml. of 28 per cent homogenate in buffer pH 7.4, each 2 mg. of ATP and DPN, 1 mg. of TPNH, final volume 3.8 ml., incubation at 38°c. for three hours in a reciprocating water bath.

TABLE VIII

Incorporation of 2-^{14}C-Mevalonic Acid into Squalene and Cholesterol (Liver Homogenate)*

Rat Livers	No. of Rats	Radioactivity Incorporated into Unsaponifiable Material (%)	Distribution of Radioactivity (%)	
			Squalene	Cholesterol
Normal (pair-fed controls)	12	15.2	12.6	81.4
Vitamin A deficient.....	12	8 3	50 7	45.0
Significance of differences between mean values:		p<0.01	p<0 01	p<0.01

* Test conditions: 3 ml. of 28 per cent homogenate in buffer pH 7.4; 400 μg. of 2-^{14}C-mevalonic acid (14.2×10^6 c.p.m.) each 2 mg. of ATP and DPN, final volume 3.8 ml., incubation at 38°c. for one hour under 100 per cent O_2 in a reciprocating water bath.

TABLE IX

Incorporation of 2-^{14}C-Mevalonic Acid into Squalene and Cholesterol (Liver Homogenate)*

Liver Homogenate of Vitamin A Deficient Rats	No. of Rats	Incorporated Radioactivity into Unsaponifiable Material (%)	Distribution of Radioactivity (%)	
			Squalene	Cholesterol
No addition.....	3	5.4	22.3	69 2
320 I.U. vitamin A acid	3	6.4	28.0	66 1

*Test conditions: 3 ml. of 28 per cent homogenate in buffer pH 7.4, 400 μg. of 2-^{14}C-mevalonic acid (14.2×10^6 c.p.m.), each 2 mg. of ATP and DPN, 1 ml. buffer resp. vitamin A acid (100 μg.) in buffer pH 7.4, final volume 4.8 ml., incubation at 38°c. for one hour under 100 per cent O_2 in a reciprocating water bath.

the three components was, however, markedly different.

In vitro studies have not proved very satisfactory up to now. First of all, we have not been able to demonstrate the *in vitro* synthesis of ubiquinone using labeled mevalonic acid, even when different possible precursors, such as the dimethoxy-methyl-benzoquinone, were added to the incubation mixture (Table VII).

On the other hand, a rather great variation of the mevalonic acid incorporation into cholesterol and squalene was observed in parallel trials. Compiling all the data on several *in vitro* experiments, an influence of vitamin A deficiency could be demonstrated by statistical evaluation. The average values for squalene are about 12 per cent in normal and 50 per cent in vitamin A deficient animals; the correspond-

TABLE X

Synthesis of Squalene, Cholesterol and Ubiquinones After Dosing Rats with 2-^{14}C-Mevalonic Acid during Increasing Vitamin A Deficiency

Days of Vitamin A Depletion	Rat Weights (gm. duplicates)		Total Radioactivity in Liver Unsaponifiable	Per Cent of Radioactivity in Different Fractions (Radioactivity of the Unsaponifiable taken as 100 Per Cent)		
				Squalene	Ubiquinone	Cholesterol
0.....	37	35	98,500	2.0	2.5	95.5
7.....	59	53	99,000	8.7	10.7	80.6
14.....	87	79	101,800	19.3	12.8	67.9
21.....	101	106	67,400	25.0	16.1	58.9
24.....	90	74	63,500	26.9	18.5	54.6
Single dose of 500 I.U. vitamin A orally per animal						
20 hours after vitamin A dose.	105	85	95,500	13.8	10.3	75.9
7 days after vitamin A dose..	131	101	83,400	11.7	8.7	79.6

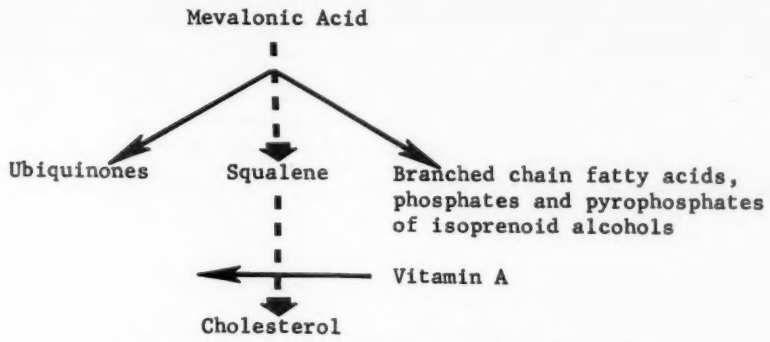

FIG. 5. Reactions necessary for biosynthesis of cholesterol.

ing values for cholesterol are 81 to 45 per cent (Table VIII).

No effect could be seen after *in vitro* addition of vitamin A acid to rat liver homogenate of vitamin A deficient rats (Table IX).

To obtain more information on the kind of interaction between vitamin A deficiency and cholesterol, squalene and ubiquinone synthesis, the incorporation of mevalonic acid was tested in the course of vitamin A deficiency (Table X).

From these results it is quite obvious that an alteration in mevalonic acid incorporation occurs during a very early stage of vitamin A deficiency. Even after a deficiency period of one week, when no signs of deficiency can be seen, the incorporation of mevalonic acid into squalene and ubiquinone is clearly increased, whereas the biosynthesis of cholesterol is depressed. By administration of vitamin A during rather severe deficiency, a clear trend to normalization was observed, even a few hours after the vitamin A dose.

According to these results it seems rather unlikely that the observed alteration in the cholesterol, squalene and ubiquinone biosynthesis is due to secondary effects of vitamin A deficiency; e.g., in the form of tissue damage. Assuming that the action of vitamin A takes place on a metabolic level, the results obtained can be explained by vitamin A being involved in one or more reactions necessary for the biosynthesis of cholesterol following the squalene formation, as indicated in Figure 5.

This would mean that in vitamin A deficiency cholesterol biosynthesis is reduced owing to a block between squalene and cholesterol. Ubiquinone would be accumulated by feedback reactions. The further observation that the radioactivity of the fraction containing the branched chain acids or the phosphates and pyrophosphates of isoprenoid alcohols is also enhanced in vitamin A deficiency, is in agreement with this hypothesis.

SUMMARY

After administration of ^{14}C-labeled mevalonic acid to rats it could be shown by isolation and chemical degradation of the biosynthetically labeled ubiquinone that mevalonic acid is the precursor of the side-chain of ubiquinone. After dosing ^{14}C-methyl labeled methionine, radioactivity was incorporated into the methoxy groups of ubiquinone in the liver of rats. No conclusive results have been obtained up to now on the precursor of the benzoquinone part of ubiquinone.

The biosynthesis of ubiquinone was found to be increased in vitamin A deficient rats, whereas that of cholesterol was depressed. Squalene is enriched in the liver of the deficient animals. This alteration in biosynthesis of cholesterol and ubiquinone takes place in a very early stage of vitamin A deficiency and can be reversed by administration of vitamin A to deficient rats even in a rather severe deficiency state. The influence of vitamin A deficiency on cholesterol biosynthesis could also be demonstrated in experiments with rat liver homogenate preparations by statistical evaluation. A possible mechanism for the interaction between vitamin A deficiency and cholesterol, squalene and ubiquinone synthesis is discussed.

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DISCUSSION

Dr. W. E. J. Phillips, (*Ottawa, Canada*): You have stressed in your paper that your observations indicate a direct effect of vitamin A *per se*, and not a secondary effect of the deficiency syndrome. I wondered how you could justify this when the effects you noted have so far been observed only in the rat, and then only in rat liver. This effect is not observed in other tissues of the rat nor in avian species and is not observed in another mammal, the guinea pig. Secondly, would you comment on the statement of Green and Diplock that vitamin A deficiency does not influence ubiquinone metabolism but the effects observed in vitamin A deficiency are a result of changes in the tocopherol levels of the liver?

Dr. Wiss: I am not aware of any species difference.

Dr. Phillips: My work with aspects of vitamin A deficiency in the rat agree completely with the work you have shown today. In the rat, vitamin A deficiency has increased the total amount of ubiquinone and the hepatic concentration of ubiquinone from 82 to 622 μg./gm. In relation to the incorporation of mevalonic acid, vitamin A deficiency has reduced the incorporation into sterols from 80 per cent of the total incorporation in the nonsaponifiable material to 43 per cent while the incorporation into squalene and ubiquinone has been increased.

Converse effects were observed in the guinea pig. There is no change in the concentration of liver ubiquinone in the guinea pig under the stress of vitamin A deficiency (normal, 47 μg./gm. liver; vitamin A deficient, 43 μg./gm. liver). Vitamin A deficiency did not block the synthesis of sterols but rather an increased incorporation of mevalonic acid was observed. Vitamin A deficiency did not increase the incorporation of mevalonic acid into the hydrocarbon fraction or ubiquinone. In view of this species difference and other factors, I feel that the effects of vitamin A deficiency on isoprenoid metabolism in the rat are secondary effects

Table I

Severe Vitamin A Deficiency and Cholesterol Synthesis

Vitamin A Treatment	Treatment	Incorporation into Unsaponifiable (%)	$\frac{\text{Incorporation into Digitonin p.p.t.}}{\text{Incorporation into Unsaponifiable}} \times 100$	Specific Activity of Digitonin p.p.t. Measured as Cholesterol (c.p.m./mg.)
−	Complete system*	38.0	12.8	32,404
−	Complete system	28.4	17.4	32,924
−	Complete system + G-1-P†	28.2	15.9	29,842
−	Complete system + G-l-P	20.1	27.2	36,510
+	Complete system	25.3	44.0	74,182
+	Complete system	21.5	26.8	38,389
+	Complete system + G-l-P	22.8	42.2	64,150
+	Complete system + G-l-P	31.8	43.6	92,377

* Complete system contained 3 ml. of 10,000 × g supernatant (pH 7.4); ATP, 2×10^{-3} mM.; DPN, 1.4×10^{-3} mM.; TPN, 1.3×10^{-3} mM.; and 2 μC. of mevalonic-2-C^{14} acid, 4×10^{-3} mM.

† Glucose-1-phosphate, 25 μM.

of the deficiency syndrome rather than a direct lack of vitamin A *per se*.

DR. G. J. WRIGHT (*Urbana, Illinois*): Work which we have done using liver enzyme preparations from severely deficient rats are in agreement with Dr. Wiss's *in vivo* work (Table I). The conversion of C^{14}-mevalonic acid into C^{14}-cholesterol was found to be depressed by vitamin A deficiency, even though our earlier work in the intact animal had shown no effect of vitamin A deficiency on incorporation of acetate into cholesterol at a stage when there was a very marked depression in incorporation of acetate into glycogen (Wolf, G., Lane, M. D. and Johnson, B. C. *J. Biol. Chem.*, 225: 995, 1957).

With mildly deficient rats we found no *in vitro* effect on cholesterol labeling from mevalonic acid even though MPS synthesis was affected in tissues from the same animals.

Vitamin A in Adrenal Hormone and Mucopolysaccharide Biosynthesis

GEORGE WOLF, D. PHIL.*

WE KNOW OF one function of vitamin A, that in vision, through the researches of Wald and his team. Through their work we know more about what this vitamin does in one particular biochemical reaction sequence than we do about any other fat-soluble vitamin. However, an animal dies of vitamin A deficiency, but not necessarily from blindness. Therefore, vitamin A must have another metabolic function. We began our search for this function in the most generalized way,[1] by comparing the metabolism of labeled acetate in vitamin A deficient and pair-fed normal rats. No differences were discernible in the rate of incorporation of radioactivity into a large variety of metabolic intermediates and products which were investigated. The only product which showed a severe depression in radioactivity incorporation was glycogen. The block caused by the deficiency was localized in the conversion of triose to glucose.[2] Table I shows the progressive loss of the capacity to incorporate radioactivity into glycogen as the deficiency proceeds, and it is to be noted that a severe drop of glyconeogenesis takes place before any other symptoms but weight loss are noticed. It was further shown that this effect was indirect,[2] and due to a lack of adrenal glucocorticoid hormones, as shown in Table II. It can be seen that the lesion is not between glucose and glycogen, but between acetate and glycogen, and that it can be corrected by cortisone injection. Histologically, as had already been indicated by Morton and his co-workers,[3] the adrenocortical lesion was seen to consist of a migration of phospholipid substances from the zona fasciculata to the zona glomerulosa. This change in the cortex begins at a time in the progress of the deficiency before even the effect on glycogen synthesis has started.

We then investigated corticosterone production by quartered adrenal glands *in vitro*. Tetrazolium-blue reducing steroids secreted into the medium were measured. Their value dropped to about one-half when using adrenal glands from animals in the earliest stages of the deficiency, and could be restored to normal by pre-injection of vitamin A as little as six hours prior to killing and excision of the glands.[4]

Next, we used adrenal homogenates incubated with labeled cholesterol and investigated its conversion to various steroids, which were isolated by paper chromatography.[5] Table III shows that a number of steroids on the pathway from cholesterol to corticosterone show lowered incorporation of radioactivity in vitamin A deficiency. If mildly deficient animals were used, however, only the last step

TABLE I

Gross Symptoms and Glycogen Biosynthesis from Acetate in Vitamin A Deficiency

Pair No.	Condition of Vitamin A Deficient Rat	Total Activity of Liver Glycogen (c.p.m.)	
		Normal	Deficient
1	No symptoms	43,500	42,300
2	No symptoms	42,200	37,600
3	Slow growth	11,300	7,060
4	Weight loss (2 days)	12,090	203
5	Severe weight loss	11,480	236
6	Severe xerophthalmia	9,930	0

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* Associate Professor of Animal Nutrition.

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TABLE II

Effect of Cortisone Treatment on Glycogen Formation in Livers of Normal and Vitamin A Deficient Rats

Percursor	Cortisone Treatment*	Total Activity of Liver Glycogen (c.p.m.)	
		Normal	Deficient
Acetate-1-C^{14}	−	28,800	2,540
Acetate-1-C^{14}	+	21,100	24,400
Glucose-1-C^{14}	−	121,200	101,200
Glucose-1-C^{14}	+	125,000	120,000

* + = rats were treated with cortisone acetate (5 mg. per day) for four days, then given 300 mg. non-labeled glucose, and thirty minutes later 7.5×10^6 c.p.m. labeled compound intraperitoneally.

TABLE III

Labeled Steroids Produced by Pig Adrenal Tissue Using Cholesterol-C^{14} as a Precursor*

Steroid	Activity Found in Deficient Tissue (d.p.m.)	Activity Found in Normal Tissue (d.p.m.)
Corticosterone	43,428	88,620
Progesterone	18,312	60,300
DOC	9,908	38,488
17 OH DOC	27,832	...†

* Tissue was homogenized and incubated in Sorenson's phosphate buffer, pH 7.2 with the following concentration of additives: sucrose, 0.025 M; KCl, 0.154M; NaCl, 0.154 M; niacinamide, 5 mM; sodium fumarate, 5 mM; $MgSO_4$, 5 mM; glucose, 0.01 M. Cofactors added: ATP, 1 mM; DPN, 0.5 mM; TPN, 0.5 mM. Incubated for two hours under 95 per cent O_2 + 5 per cent CO_2. Values represent an average of three experiments. 1.25 μc. cholesterol-4-C^{14} added. Total volume 3 ml.

† Undetectable.

TABLE V

Corticosterone Produced Under Conditions of Increased TPNH Production*

Addition	Corticosterone Produced in Deficient (d.p.m.)	Corticosterone Produced in Normal (d.p.m.)
Homogenate	2,650	7,548
Homogenate + glucose-6-phosphate	3,104	8,552
Homogenate + glucose-6-phosphate + glucose-6-phosphate dehydrogenase	2,412	9,196

* Conditions of incubation were the same as those given in Table III. Cofactors present at following concentration: ATP, 1 mM; DPN, 0.5 mM; TPN, 0.5 mM; glucose-6-phosphate, 0.5 mM. Three units of glucose-6-phosphate dehydrogenase were added.

in the sequence, the conversion of desoxycorticosterone to corticosterone, is so affected (Table IV). Table V shows that this depression is not due to lack of the essential coenzyme in this reaction, reduced triphosphopyridine nucleotide (TPNH). As is evident from the data in Table VI this lesion can readily be corrected by addition of vitamin A, as well as vitamin A acid, to the homogenate.

To summarize, then, we have traced an abnormal metabolism of acetate in vitamin A deficiency to a participation of the vitamin in the biosynthesis of corticosterone, apparently in the hydroxylation of desoxycorticosterone, and presumably a direct participation, since the vitamin is effective by direct addition to adrenal homogenate *in vitro*.

The outward symptoms of vitamin A de-

TABLE IV

Labeled Steroids Produced when Rat Adrenal Tissue Was Incubated with Cholesterol-C^{14}

Enzyme Source and Addition	Mildly Deficient		Normal	
	Corticosterone (d.p.m.)	Progesterone (d.p.m.)	Corticosterone (d.p.m.)	Progesterone (d.p.m.)
1. Whole homogenate	2,190	...	3,660	...
1. Mitochondria	...	11,538	...	9,312
2. Mitochondria	...	5,996	...	3,160
2. Mitochondria + progesterone	...	3,856	...	2,094

NOTE: Conditions of incubations as given in Table III.

TABLE VI

Corticosterone Production from Cholesterol-4-C^{14}*

	C^{14} Activity in Corticosterone in Deficient Tissue (d.p.m.)	C^{14} Activity in Corticosterone in Normal Tissue (d.p.m.)
Whole homogenate	9,630	13,706
Whole homogenate + vitamin A	14,998	†
Whole homogenate + vitamin A acid	16,576	†

* Vitamin A added at level of 1.3 μM/incubation. DPN and TPN, 0.5 mM; ATP, 1 mM. 2.2×10^6 d.p.m. cholesterol added to each incubation. Incubated for one hour under 95 per cent O_2, 5 per cent CO_2.

† Not determined.

TABLE VII

Incorporation of $S^{35}O_4^=$ into Mucopolysaccharides by Colon Homogenates

Vitamin A Status	Vitamin A Added (10 μg.)	Mucopolysaccharide (c.p.m.)
−	−	346 (3)
−	+	805 (2)
+	−	936 (4)
−	serum-albumin-ethanol	268 (2)

NOTE: The values are averages of the number of incubations shown in parentheses. The complete system contained 10 μM of ATP, 6 μM of Mg^{++}, 1 μM of glutamine, 3 μM of DPN and 10 μM of glucose: final volume, 1 ml. Each incubation contained 10.6×10^6 c.p.m. of radioactive sulfate and 14 mg. of protein. + = normal rat; − = deficient rat.

TABLE VIII

Effect of Vitamin A on the Incorporation of $S^{35}O_4^=$ into Mucopolysaccharides by Colon Homogenates Containing UDP-Derivatives

Vitamin A Status	Addition of Vitamin A (10 μg.)	Mucopolysaccharides (c.p.m.)
+	−	832 (2)
−	−	270
−	+	681

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 1 μM of UDPGA, 1 μM of UDPG, 1 μM of glutamine, 8 μM of AG (all obtained from Sigma Chemical Corporation), 1 μM of ATP and 10 μM of Mg^{++}. Each incubation contained 8.5×10^6 c.p.m. of activity and 14 mg. of protein. + = normal rat; − = deficient rat.

TABLE IX

Incorporation of S^{35}-Sulfate Into Mucopolysaccharide by Subcellular Fractions and pH 5 Enzymes of Pig Colon Mucosa

Subcellular Fraction	Mucopolysaccharide (c.p.m./mg. protein)
Supernatant free from mitochondria and nuclei	1,293 (4)
Supernatant free from mitochondria, nuclei and microsomes	1,521 (4)
Supernatant free from mitochondria, nuclei, microsomes and pH 5 enzymes	326 (1)
Microsomes	516 (4)
pH 5 Enzymes	1,728 (4)
pH 5 Enzymes and microsomes	1,770 (3)

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 10 μM ATP, 6 μM $MgCl_2$, 3 μM DPN, 1 μM glutamine and 10 μM glucose. The radioactivity added per incubation, 9×10^6 c.p.m. made to final volume of 1 ml. with phosphate buffer, pH 7.4.

ficiency show no similarity to those found in cases of adrenal insufficiency, and, indeed, the only detectable consequence of the glucocorticoid hormone lesion is the decreased capacity for glycogen synthesis. Therefore, the adrenal cortex disturbance must be only one of a number of divergent symptoms of vitamin A deficiency, the most apparent of which is the deterioration of mucous membranes. We were led, therefore, to an investigation of the influence of vitamin A on the metabolism of mucopolysaccharides, which are the characteristic constituents of mucus. Already in 1953,[6,7] H. B. Fell had shown that the profound effect of vitamin A on another type of mucopolysaccharide, that of bone and cartilage, is direct and not mediated through a hormone. We found the most promising tissue for *in vitro* synthesis of MPS (mucopolysaccharide) to be the mucosa of colon. Investigating the uptake of labeled sulfate into MPS, we observed,[8] both in colon segments and homogenates, a depression of MPS synthesis in vitamin A deficiency, restorable specifically by vitamin A added to the medium (Table VII). Figure 1 shows the pathway of MPS synthesis, as now accepted. By using, as substrates in MPS synthesis, intermediates closer to the

TABLE X

Effect of Preincubation with Lipoxidase on the Incorporation of $S^{35}O_4^=$ Into Mucopolysaccharide Synthesized by pH 5 Enzymes

Lipoxidase (0.3 mg./ml.)	Added Vitamin A (10 μg.)	Mucopolysaccharide (c.p.m./mg. protein)
–	–	2,706 (4)
0.1	–	1,213 (4)
0.1	+	2,188 (4)

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 10 μM of glucose, 1 μM of glutamine, 3 μM of DPN and $S^{35}O_4^=$, 12 × 10^6 c.p.m., made up to 1 ml. with phosphate buffer, pH 6.8.

final product, such as galactosamine, glucosamine 6-phosphate, uridine diphosphoglucuronic acid, and uridine diphosphoacetylglucosamine, we could still obtain the effect of vitamin A, as is illustrated, for instance, in Table VIII. It was obvious, therefore, that the vitamin was not involved in the biosynthesis of any of these intermediates. We were able to trace the capacity for MPS synthesis to an enzyme fraction obtainable from pig colon mucosa,[9] where it resided in the protein fraction of the supernatant solution after high speed centrifugation, precipitable at pH 5.2 (Table IX). This enzyme fraction is again susceptible to vitamin A deficiency, as well as to lipoxidase treatment (Table X). Lipoxidase is known to destroy vitamin A, and it lowers MPS synthesis in the pig colon mucosal enzyme fraction. The activity can again be restored by added vitamin A.

Up to this point we have been investigating only the incorporation of infinitesimally small amounts of labeled substrates (sulfate or glucose) into MPS. It was necessary to determine whether net synthesis also was affected by vitamin A. We were able to show that colons of vitamin A deficient rats contained 40 per cent less total hexosamines than those of normal rats and that this was due to a deficiency of galactosamine rather than of glucosamine.[10] We next developed a cell-free mucosal homogenate system[11] which, with glucose 6-phosphate, uridine and adenosine triphosphates and diphosphopyridine nucleotide, gave net synthesis of total and of mucopolysaccharide-bound hexosamines. Net synthesis was about 1 μM MPS-bound hexosamine per 100 mg. protein, lowered to about 0.3 μM in deficiency and restorable with added vitamin A (but no other fat soluble vitamin).

The minimum effective amount was determined to be 1.25 × 10^{-2} μM vitamin A per incubation.

As mentioned above, by the use of substrates in MPS synthesis which were closer to the final product, an analysis could be made of the particular reaction which would be susceptible to vitamin A deficiency. None was found, until incubations were attempted with S^{35}-labeled phosphoadenosine phosphosulfate (PAPS, "active sulfate") instead of with sulfate (see Figure 1). It could be shown[12] that, whereas vitamin A deficiency severely lowered sulfate incorporation into MPS from free sulfate, no such depression was obtained with the use of PAPS. As shown in Table XI, vitamin A deficiency causes lowered PAPS synthesis, a reaction

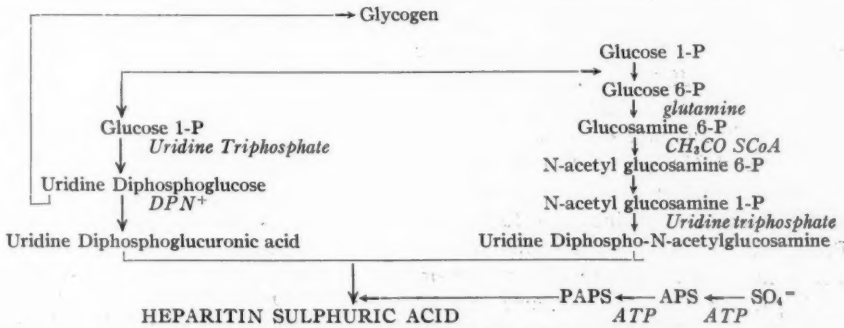

FIG. 1. Possible pathway of mucopolysaccharide biosynthesis (P, phosphate; CH_3CO-SCoA, acetyl coenzyme A; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate APS, 5′-adenosinephosphosulfate).

TABLE XI
Effect of Vitamin A Deficiency on $PAPS^{35}$ Synthesis

Vitamin A Status of Rats	Addition	$PAPS^{35}$ (c.p.m./mg. protein)	
		Experiment 1	Experiment 2
Adequate	...	19,200	59,000
Deficient	...	8,440	28,700
Deficient	Vitamin A, 20 μg. in propylene glycol, 5 μl.	24,300	54,100
Deficient	Propylene glycol, 5 μl.	...	31,400

Activity added: Experiment 1, 8.4×10^5 c.p.m.; Experiment 2, 16.8×10^5 c.p.m.

which is an essential step in the formation of sulfated MPS. The lesion was again restorable to normal with added vitamin A. It seems, therefore, that the capacity of vitamin A to affect MPS synthesis lies in its function in the activation reactions of sulfate. Preliminary evidence would implicate the first step; i.e., the reaction of adenosine triphosphate with sulfate to give adenosine phosphosulfate, as being vitamin A dependent. Isolation of the enzyme involved, and a search for vitamin A or a derivative thereof in the enzyme, is now under way.

It is possible, albeit somewhat speculative, to generalize from these observations. As Fell has shown, excess vitamin A causes a dissolution of the MPS part of cartilage matrix, presumably by a breakage of the MPS-protein bound in cartilage. On the other hand, vitamin A stimulates the formation of MPS of mucus. We are led to the hypothesis that the activation of sulfate is the rate limiting step in MPS synthesis. Sufficient vitamin A leads to a maximum rate of MPS synthesis. Large amounts of MPS, in some unexplained way, cause dissolution of the MPS-protein bound in cartilage and the increased formation of MPS bound in mucus. The concentration of MPS itself may be the regulatory factor determining whether it is to be bound in cartilage or in mucus. Future research will show whether or not these hypotheses are borne out in fact.

SUMMARY

Vitamin A deficiency was found to cause an abnormal metabolism of acetate, and reduced glyconeogenesis. This was traced to a malfunction of the adrenal cortex and, ultimately, to an interruption of adrenal glucocorticoid hormone (corticosterone) biosynthesis in the vitamin A deficient rat. In adrenal cortex homogenates from vitamin A deficient animals, the reduced corticosteroid biosynthesis could be restored to normal by adding vitamin A or vitamin A acid *in vitro*. The influence of the vitamin on one of the enzymes of corticosteroid biosynthesis seems, therefore, to be a direct one.

Another lesion caused by vitamin A deficiency, the degeneration of mucous membranes, was found to be due to a requirement for vitamin A in mucopolysaccharide biosynthesis. This was shown by lowered incorporation of labeled sulfate or glucose into mucopolysaccharide in rat colon homogenates, restorable specifically by added vitamin A. Similarly, net synthesis of mucopolysaccharides in rat colon homogenates, as measured by an increase in mucopolysaccharide-bound hexosamines, is partially dependent on vitamin A. This effect was traced to an enzyme fraction and finally to a single reaction in the biosynthesis of mucopolysaccharide, the activation of sulfate to form adenosine phosphosulfate.

ACKNOWLEDGMENT

The participation of the following co-workers, past and present, on this project, is gratefully acknowledged: Drs. B. C. Johnson, S. G. Kahn, M. D. Lane, S. R. Wagle, R. A. Van Dyke, G. J. Wright, P. T. Varandani, M. L. Chang and A. Moretti and Mr. J. G. Bergan.

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DISCUSSION

DR. JOHN. A. LUCY (*Madison, Wisconsin*): Dr. Wolf has demonstrated that the *in vitro* incorporation of (^{35}S) sulphate into mucopolysaccharide by segments and homogenates of colon from vitamin A deficient rats is reduced as compared with the incorporation observed in control experiments with tissue from normal animals. Furthermore, the point of action of the vitamin appears to be at the stage of polymerisation of uridine diphosphate derivatives or in the transfer of sulphate to the polymer. In an investigation on the effect of excess vitamin A on sulphate incorporation in a different tissue, embryonic cartilage, Fell, Mellanby and Pelc found that limb-bone rudiments of the chick cultured *in vitro* in the presence of vitamin A first ceased to take up labeled sulphate and then lost that already incorporated. The loss of (^{35}S) sulphate from the matrix was accompanied by the disappearance of metachromasia. Dr. Honor B. Fell, John T. Dingle and I have been studying the mode of action of this effect of excess vitamin A on cartilage and have obtained evidence that loss of metachromasia results from an effect on the protein of the tissue rather than on the metabolism of the polysaccharide.

We have observed that after six days' cultivation in medium containing added vitamin A(10 i.u./ml.) the hexosamine content of the limb-bone rudiments is reduced to half that of the controls grown on normal medium. It was also found that degradation of the protein of the culture medium (plasma: embryo extract clot) was greater in the vitamin A treated cultures than in the control cultures. Experiments with normal embryonic cartilage demonstrated that treatment of the rudiments with distilled water at 4°c. for one hour followed by incubation in buffer for two hours at 37°c. at pH 3-5 results in a loss of metachromatic staining properties and liberation of polymeric hexosamine-containing components into the buffer. During the water treatment, a proteolytic enzyme that had an acid pH optimum was released from cytoplasmic particles of the cartilage tissue. In experiments with rat liver lysosomes that were suspended in 0.25 M. sucrose, it has been demonstrated that a proteolytic enzyme is released on the addition of vitamin A; the pH optimum of the enzyme liberated was the same as that of the enzyme obtained by hypotonic treatment. These observations indicate that a possible mechanism for the action of excess vitamin A on cartilage may involve alteration of the permeability of lysosomes and other intracellular particles. Proteolytic enzymes may be released in this way which then degrade cartilage matrix with a resultant loss of polysaccharide.

While Dr. Wolf has observed that addition of vitamin A stimulates the incorporation of sulphate into mucopolysaccharide by rat colon tissue, we have found that excess vitamin A appears to act on cartilage by stimulating proteolytic degradation of the protein-polysaccharide complex of the matrix. The difference in response of these two tissues to the vitamin in our respective experiments seems to be of a qualitative rather than quantitative nature and this difference may reflect two distinctive patterns of enzyme distribution in these tissues. The response of a particular tissue that is susceptible to vitamin A may depend upon the relative

TABLE I

Reproduction in the Vitamin A Deficient Rabbit

Weeks on Diet	Treatment	No. Mated	Average Young		Sites	Vitamin A	
			Living	Dead		Liver (γ/gm.)	Plasma (γ/100 ml.)
12	None	14	0.9	0.6	1.5	1.1	4.4
12	Progesterone	14	4.8	2.9	0.1	1.1	5.7

TABLE II

Reproduction in Rats Fed Only Sucrose and Distilled Water during Gestation

Diet	Treatment	No.	Average Young	
			Living	Dead or Sites
Control	None	108	10.3	0.4
Sucrose	None	35	1.2	7.7
Sucrose	Progesterone	33	6.2	2.9
Sucrose	Prolactin	5	7.8	2.2

quantities of certain enzymes in the tissue which are stimulated by the vitamin either directly, or indirectly by a mechanism such as that proposed for the action of excess vitamin A on cartilage.

DR. RAY L. HAYS: In the broader aspects of the effect of vitamin A or the lack of it on the well being of the animal, it was interesting to find exogenous progesterone an aid in alleviating some of the effects of a vitamin A deficiency on reproduction in the rabbit. Table I shows the results of our experiments.[1] It can be noted that while the liver and plasma vitamin A values are extremely low, reproduction was greatly improved by the daily injection of 8 mg. of progesterone. In the progesterone treated animals there were more living fetuses and even those which died had lived longer than those of the untreated females.

Table II shows the result of further work we have done in this area. When rats were placed on a diet of sucrose and distilled water for the duration of pregnancy, the average number of living young was reduced to 1.2.[2,3] This detrimental effect of the severely restricted diet was mostly alleviated by daily injections of 5 mg. of progesterone or 4-6 I.U. of prolactin.[4]

It would seem that the feeding of only sucrose caused a deficiency in the secretion of prolactin by the anterior pituitary, which caused a decrease in the progesterone secretion and the resultant loss of fetuses. It is possible that a similar mechanism is involved in vitamin A deficiency. On the other hand, the level of vitamin A in the blood may have been too low to permit normal essential physiologic function and that progesterone in some way compensated for it.

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The Role of Quinones in the Mitochondrial Electron Transport System

DANIEL M. ZIEGLER, PH.D.*

OF THE NATURALLY occurring quinones that have been considered as possible electron carriers in the mitochondrial electron transport system, only coenzyme Q (CoQ) has been unambiguously shown to function in this capacity and this report will be concerned primarily with the role of CoQ in mitochondria. Prior to the discovery of CoQ, reports by Nason and his co-workers[1,2] suggested that α-tocopherol might function as an electron carrier in the mitochondrial electron transport system. The following is a brief summary of our current views on the role of tocopherol in the mitochondrial electron transport system.

Hatefi and his associates in our laboratory have examined the tocopherol content of beef heart mitochondria and of submitochondrial particles and some of their data is given in Table I. In agreement with the earlier reports from the laboratories of Nason[2] and of Slater,[3] substantial quantities of α-tocopherol are present in mitochondria and its distribution would suggest that it is closely associated with the reduced diphosphopyridine nucleotide (DPNH) oxidase system. In contrast to total lipid and CoQ, the level of α-tocopherol is almost twice as high in the purified DPNH cytochrome *c* reductase preparation as in the original mitochondria and it is evident that concentration of tocopherol parallels concentration of DPNH cytochrome *c* reductase activity. Whether this has any physiologic significance or is merely a reflection of the methods used to isolate the particle remains to be determined. So far we have not been able to detect cyclic oxidation and reduction of tocopherol during oxidation of DPNH, and it appears unlikely that the quinone to which it gives rise functions as an electron transport compound in the DPNH oxidase system. If tocopherol has a role, other than that of a lipid antioxidant,[4,5] it has yet to be clearly demonstrated in isolated mitochondria, and at the present time we cannot assign a more specific function to tocopherol. However, when we fully understand the role of the other mitochondrial lipids in electron transport[6] we may be able to define more clearly the role of tocopherol.

In contrast to tocopherol, CoQ does undergo cyclic oxidation and reduction during the oxidation of succinate or DPNH.[7–9] The evidence that this quinone is an obligatory electron transport component of the terminal electron transport system has been well documented and is summarized in recent reviews by Green.[6,10] Since most researchers in this field are probably familiar with the earlier work on CoQ, I have omitted the details. Instead, I shall comment on some of our recent experiments dealing with the mechanism of CoQ reduction in heart mitochondria.

POSITION OF COENZYME Q IN THE ELECTRON TRANSPORT CHAIN

It is generally accepted that CoQ is positioned between the flavoproteins and cytochrome c_1 and is required to link the flavoproteins to the cytochromes.[11,12] Reduced CoQ is reoxidized by cytochrome c_1 and at least in nonphosphorylating systems, cytochrome *b* is not an obligatory electron carrier either in the reoxidation of CoQ or in the reduction of the quinone by substrate.[11] However, the relative positions of CoQ and cytochrome *b* in phosphorylating preparations remain to be

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* Established Investigator.

Part of this work was carried out during tenure as an Established Investigator of the American Heart Association.

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TABLE I

Tocopherol Content of Heart Mitochondria and the DPNH-cytochrome *c* Reductase Particle*

Preparation	Total Lipid (mg./mg. protein)	CoQ mμM / mg. protein	Tocopherol mμM / mg. protein
Beef heart mitochondria	0.37	3.5	0.97
DPNH-cytochrome *c* reductase particle†	0.33	3.7	1.56

* Unpublished data of Hatefi, Y., Haavik, A. G. and Jurtshuk, P.

† This preparation was isolated by the method described by Hatefi et al.[23] The specific activity was 60 μM cytochrome *c* reduced per min. mg. protein at 38°c.

determined. The problem of the function of cytochrome *b* lies outside the scope of this report and I shall confine my comments to the mechanism of CoQ reduction in nonphosphorylating preparations. We cannot rule out the possibility that in mitochrondria, CoQ is reduced by an alternate pathway involving cytochrome *b*, but on the other hand, the evidence for such a pathway is far from convincing.

CoQ does not react directly with the flavoproteins since neither the primary succinic flavoprotein[13] nor the lipoflavoprotein diaphorase[14] is able to catalyze the reduction of CoQ. It is apparent that an additional component is required to link the flavoprotein to CoQ. By purifying the succinic CoQ reductase of heart mitochrondria we have been able to define the components required for the enzymatic reduction of CoQ, but before I comment on the properties of the isolated enzyme I would like to describe the methods we use to measure succinic CoQ reductase activity.

METHODS OF ESTIMATING SUCCINIC CoQ REDUCTASE ACTIVITY

Coenzyme Q_{10}, the naturally occurring quinone, is practically insoluble in water and as such cannot be used as a final electron acceptor. However, the effective concentration of CoQ_{10} in aqueous solution can be increased many fold by dispersing the quinone with small quantities of nonionic detergents. The amount of detergent required to disperse CoQ_{10} does not affect

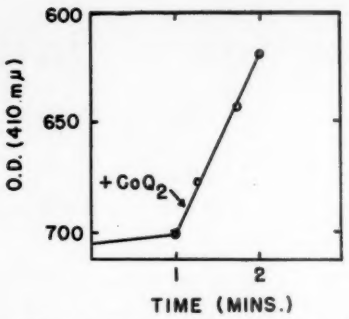

FIG. 1. Effect of CoQ_2 on the succinic ferricyanide reaction. The reaction medium contained 100 μM phosphate, pH 7.0, 0.7 μM potassium ferricyanide, 0.1 μM Versene and 3.0 μM succinate per ml. The reaction was started by adding enzyme and the rate of ferricyanide reduction was measured at 410 mμ. After one minute 1 μL. CoQ_2 (5 μM per ml. in ethanol) was added.

the succinic dehydrogenase, and an assay system for measurement of succinic CoQ_{10} reductase activity using CoQ_{10} emulsified with Triton® X-100 has been described.[15] The DPNH dehydrogenase is far more sensitive to detergents and the conditions necessary to obtain rapid reduction of substrate amounts of CoQ_{10} have not as yet been found.

While CoQ_{10} can be used as the final electron acceptor with the succinic dehydrogenase, the succinic CoQ reductase activity of mitochondrial preparations can be more readily estimated by measuring spectrophotometrically the reduction of either 2-6-dichloroindophenol or ferricyanide. Neither of these dyes is reducible by the succinic flavoprotein[16] at concentrations below 1 μM per ml. at pH 7.0, but they are rapidly reducible by reduced CoQ nonenzymatically. The earlier report of Crane et al.[17] suggested that CoQ may be required for the rapid reduction of ferricyanide and this has been confirmed by studying the effect of CoQ on the succinic-ferricyanide reaction catalyzed by a submitochondrial particle free of endogenous CoQ (Fig. 1). The quinone stimulates the rate of ferricyanide reduction about eightfold which indicates that it can act as an intermediate electron carrier between the flavoprotein and the dye.

The succinate-ferricyanide or the succinate-2,6-dichloroindophenol reactions provide a convenient and rapid spectrophotometric method for estimating the succinic CoQ reductase activity of mitochondrial preparations.

TABLE II

The CoQ Reductase Activities and the Turnover Rate of the Succinic Flavoprotein in Mitochondria and the Two Soluble Preparations

Preparation	CoQ Reductase Activities ($\frac{\mu M \text{ CoQ reduced}^*}{\text{minute/mg. protein}}$)	Turnover Rate† of Flavoprotein		
		Phenazine Methosulfate	CoQ	O_2
Beef heart mitochondria	1.1	9,800	10,000	9,100
Succinic CoQ reductase[19]	56.0	11,300	12,600	...
Primary succinic flavoprotein[13]	0.0	...	4,100	...

* 2-6-dichloroindophenol assay.

† The turnover rate is expressed as moles of succinate oxidized per minute per mole of succinic flavin with CoQ, phenazine methosulfate and oxygen as final electron acceptors.

In these assays the quinone is used in catalytic amounts and the water insolubility of CoQ_{10} is not a serious problem since 10 mμM of CoQ_{10} per ml. is usually sufficient to saturate the enzyme. This concentration of the quinone in water can be readily achieved by adding 20 to 60 μgm. of Triton X-100 per ml. to the assay medium. The detergent is not necessary when CoQ_2 is used instead of CoQ_{10}, since CoQ_2 is considerably more water soluble than the naturally occurring quinone.

Either 2,6-dichloroindophenol or ferricyanide can be used as the final electron acceptor in these assays. However, 2,6-dichloroindophenol is more suitable with crude enzyme preparations since it does not react directly with the primary succinic flavoprotein.[16] By contrast, ferricyanide, under certain conditions,[18] can react directly with the flavoprotein, and the reaction becomes CoQ independent.

PROPERTIES AND COMPOSITION OF THE PURIFIED SUCCINIC COENZYME Q REDUCTASE

With the aid of the spectrophotometric assay method a soluble form of the succinic dehydrogenase that contains all the components required to catalyze the rapid reduction of CoQ by succinate was isolated in our laboratory.[19] This enzyme will be referred to as the succinic CoQ reductase to distinguish it from the primary succinic flavoprotein isolated earlier by Singer et al.[13] In Table II is listed the relative dehydrogenating activities of the two forms of the dehydrogenase. Both forms of the flavoprotein catalyze the reduction of phenazine methosulfate, but only the reductase can react with CoQ, and the isolated enzyme is about fifty times more active than heart mitochondria in catalyzing the reduction of CoQ (Table II).

The turnover of the flavoprotein (Table II) in the succinic CoQ reductase is slightly greater than it is in the electron transport particle. This demonstrates that the components required to link the flavoprotein to CoQ are fully preserved in the isolated enzyme.

It is evident from the composition data (Table III) that the CoQ reductase is a more complex form of the succinic dehydrogenase than the primary flavoprotein. Both forms of the dehydrogenase contain 4.2 to 4.5 mμM flavin per mg. protein and in both, the flavin is released by acid only after the enzyme is treated with a proteolytic enzyme. In contrast to the primary succinic flavoprotein the CoQ reductase contains lipid and heme. The nonheme iron content of the reductase is twice that of the Singer flavoprotein. The heme component (cytochrome *b*) associated with CoQ reductase is not reduced by succinate (Fig. 1)

TABLE III

Composition of the Primary Succinic Flavoprotein and the Succinic CoQ Reductase

Preparation	Component			
	Flavin ($\frac{\text{m}\mu\text{M}}{\text{mg. protein}}$)	Nonheme Iron ($\frac{\text{m}\mu\text{M}}{\text{mg. protein}}$)	Heme ($\frac{\text{m}\mu\text{M}}{\text{mg. protein}}$)	Lipid ($\frac{\text{mg.}}{\text{mg. protein}}$)
Primary succinic flavoprotein*	4.2	18	0	0
Succinic CoQ reductase	4.3	35	4.6	0.18

* The flavoprotein isolated by the method of Singer et al.[13]

TABLE IV
The Reduction of Nonheme Iron in Mitochondrial Subfractions*

Preparation	Total Nonheme Iron (mμM/mg. protein)	Per cent Total Iron Reduced	
		By DPNH	By Succinate
Succinic dehydrogenase[13]	18	0	0
Succinic CoQ reductase[19]	35	0	23
Succinic-cytochrome *c* reductase[22]	13	< 1	23
DPNH-cytochrome *c* reductase[23]	15	30	< 2
Electron transport particle[28]	9	39	27

* The reduction of the nonheme iron was followed by the method of Ziegler and Doeg.[27]

TABLE V
Effect of Inhibitors on the Reduction of Nonheme Iron in the Electron Transport Particle

Inhibitors	Concentration	Per cent of Total Iron Reduced	
		By DPNH	By Succinate
None	...	39	27
Amytal	1×10^{-3} M	0	59
Malonate	2×10^{-2} M	65	0

TABLE VI
Inhibition of Succinic-CoQ and Succinic Phenazine Methosulfate (PMS) Reductase Activity by 2-Thenoyltrifluoroacetone

Preparation	Per cent Inhibition at 10^{-4} M	
	CoQ	PMS
Primary succinic flavoprotein[13]	..	0.0
Succinic CoQ reductase[19]	98	17
Succinic cytochrome *c* reductase[22]	96	12

which makes its participation as an electron carrier between flavoprotein and CoQ most unlikely. Some of the other properties of the heme component will be discussed later but first let us consider another major difference between the two forms of the succinic dehydrogenase. The nonheme iron in the CoQ reductase is functionally intact, i.e., it is reduced by succinate (Table IV); whereas, as shown earlier by Massey,[20] the nonheme iron present in the primary succinic flavoprotein is not reduced by succinate. In the CoQ reductase the ratio of iron, rapidly reduced by succinate, to flavin is close to 3:1. Since the ratio of total nonheme iron to flavin is 8:1, apparently only part of the iron functions as an electron carrier between the flavoprotein and CoQ. The electron paramagnetic spectra of CoQ reductase published by Beinert and Sands[21] also indicate that the enzyme contains at least two forms of a paramagnetic species, only one of which is reduced by succinate and reoxidized by CoQ. The "nonfunctional" iron does not seem to be extraneous iron adsorbed by the enzyme during its isolation, since the ratio of total iron to flavin is quite constant from one preparation to another. This ratio, furthermore, remains unchanged after treating the enzyme with Dowex® A-1 chelating resin. However, half of the nonheme iron can be removed by prolonged aerobic dialysis against Versene,® but the CoQ reductase activity of the preparation is irreversibly destroyed by this procedure.

The nonheme iron undergoes cyclic oxidation and reduction in other preparations capable of catalyzing the reduction of CoQ (Table IV). Succinate, but not DPNH reduces significant amounts of iron in the succinic cytochrome *c* reductase particle prepared from heart mitochondria by the method of Green and Burkhard.[22] This preparation does not contain a functional DPNH chain and cannot catalyze the reduction of CoQ by DPNH; whereas, in the DPNH cytochrome *c* reductase particle[23] which is essentially free of the succinic dehydrogenase, only DPNH reduces the nonheme iron. In the electron transport particle (ETP) where both the DPNH and succinic chains are intact either substrate can reduce between 25 to 35 per cent of the iron; however, when both substrates are added approximately 70 per cent of the total nonheme iron is reduced.

Inhibitors which specifically block either the DPNH oxidase or succinoxidase activity of ETP (Table V) have a pronounced effect on the

TABLE VII
Effect of 2-Thenoyltrifluoroacetone on the Reduction of Nonheme Iron by Succinate

Preparation	Per cent Inhibition at 10^{-3} M
Succinic CoQ reductase[19]	84
Succinic cytochrome *c* reductase[22]	91
Electron transport particle[28]	90

reduction of nonheme iron. Amytal,® a specific inhibitor of DPNH oxidase activity, completely blocks the reduction of iron by DPNH, but more iron is reduced by succinate in the presence of Amytal than by succinate alone (Table v). Malonate, a specific inhibitor of succinoxidase, prevents the reduction of iron by succinate, but increases the amount reduced by DPNH. It is difficult to interpret these phenomena with the limited amount of information available, but they do demonstrate that in the absence of inhibitors the nonheme iron compounds associated specifically with the DPNH and succinic flavoproteins do not freely intercommunicate.

The iron chelate compound, 2-thenoyltrifluoroacetone (TTA) is one of the best selective inhibitors of succinic CoQ reductase activity (Table vi).[24] The level of inhibitor required to block the reduction of CoQ has only a slight effect on the succinate-phenazine reaction catalyzed by the CoQ reductase and does not affect at all the phenazine reductase activity of the primary succinic flavoprotein. Since TTA blocks the reduction of CoQ, but not the reduction of phenazine it cannot be acting on the flavin and must act on a component between the flavin and CoQ. TTA also prevents the reduction of nonheme iron in the CoQ reductase (Table vii) and it is probable that TTA inhibits the over-all succinate to CoQ reaction by combining with the iron compound associated with the succinic dehydrogenase.

Spectra of the CoQ reductase (Figs. 2 and 3) also suggest that the enzyme contains a component in addition to the flavin that is reduced by succinate and reoxidized by CoQ. In addition to the band at 450 mμ, succinate also bleaches components that adsorb at about 480 and 415 mμ. Even at 450 mμ the changes in the spectrum produced by succinate or hydrosulfate cannot be entirely due to the flavin. Even if all of the flavin is reduced by succinate,

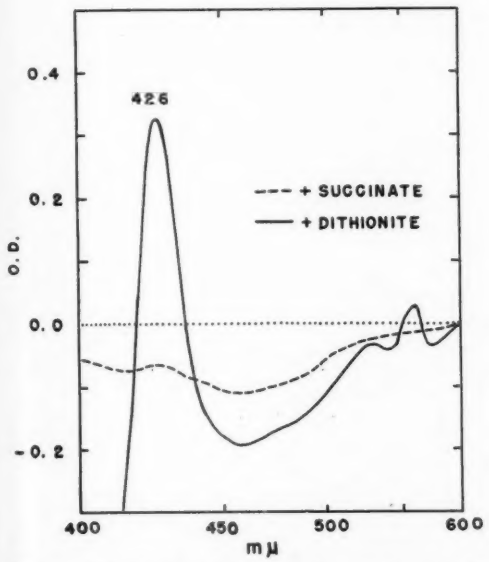

FIG. 2. Difference spectra of solutions of the succinic CoQ reductase recorded with a Beckman DK-2 spectrophotometer. The enzyme (1.8 mg./ml.) was dissolved in 0.1 M phosphate buffer, pH 7.0 and first reduced by succinate and then with dithionite.

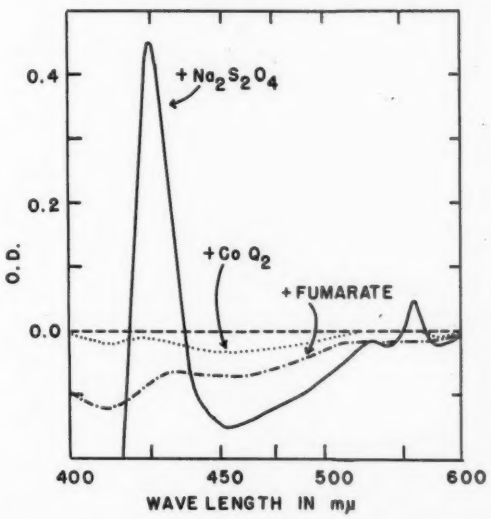

FIG. 3. Reoxidation of reduced F_s(b) with fumarate and CO_2. Difference spectra of solutions of the succinic CoQ reductase. The enzyme was first reduced by adding limiting amounts of dithionite and then reoxidized first by adding fumarate (final concentration .02 M) and CoQ_2 (final concentration 10^{-4} M).

the decrease in optical density at 450 mμ is greater than could be attributed to this component alone.

Fumarate only partially reoxidizes the reduced enzyme, reduced with dithionite (compare with Fig. 3), and it is unlikely that any of the bands remaining after the addition of fumarate can be attributed to the flavin prosthetic group. The components of the enzyme that remain reduced in the presence of excess fumarate are, however, reoxidized by CoQ (Fig. 3).

The spectral changes in the transition from the reduced to oxidized forms of the enzyme which cannot be attributed to the flavin are probably due to the nonheme iron since the nonheme iron is the only component of the enzyme other than the flavin known to be reduced by succinate and reoxidized by CoQ.

The function of the heme associated with the flavoprotein is not known. It is not reduced by succinate so it is not an obligatory electron carrier between the flavin and CoQ, but as yet it has not been possible to remove the heme without removing at the same time the lipid and some of the nonheme iron and these latter two components appear to be necessary for CoQ reductase activity. Since the reduced heme is rapidly reoxidized by fumarate (Fig. 3) a functional link still exists between the flavin and heme prosthetic groups. However, the potential of the heme must be considerably more negative than that of the flavoprotein since succinate even at a final concentration of 0.5 M. does not reduce the heme.

The CoQ reductase is not a simple mixture of a hemoprotein and a flavoprotein since separation of the two compounds cannot be achieved by the usual physical methods used to fractionate proteins. The molecular weight of the isolated enzyme as determined by measurement of sedimentation velocities corresponds very closely to the minimum molecular weight based on the flavin or heme content which suggests that the succinic dehydrogenase as a flavohemoprotein similar to the yeast lactic dehydrogenase.[25,26]

SUMMARY

Two quinones, α-tocopherol and coenzyme Q are present in high concentration in mitochondria isolated from mammalian tissues; however, only coenzyme Q has been shown to undergo oxidation and reduction during electron transport.

Coenzyme Q functions as an intermediate electron carrier between the flavoprotein and cytochrome regions of the terminal electron transport system. Coenzyme Q does not, however, react directly with the flavoproteins and evidence obtained from studies on the isolated succinic coenzyme Q reductase suggests that an additional redox component is required to mediate the transfer of electrons from the flavoprotein to coenzyme Q. This component has not been thoroughly characterized but the preliminary studies indicate that it is an iron-containing compound (nonheme) in which the iron undergoes cyclic oxidation and reduction during the oxidation of succinate by coenzyme Q.

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The Possible Function of Quinols, Chromanols and Chromenols in Intracellular Respiration

E. C. SLATER, M.SC. (Melb.), SC.D. (Contab.)*

FOUR BIOCHEMICALLY important groups of compounds—(1) the vitamins E, (2) the vitamins K, (3) the ubiquinones (or coenzymes Q), and (4) plastoquinone;—are chemically closely related (Fig. 1). The vitamins K and the ubiquinones are fully substituted *p*-quinones with one long aliphatic side chain, while α-tocopherol (the main vitamin E of animal tissues) yields a similar *p*-quinone on oxidation with $FeCl_3$ or $AuCl_3$. The other tocopherols also give *p*-quinones under these conditions but like plastoquinone (found in chloroplasts), these quinones have one or more free hydroxyl atoms in the benzene ring.

There are some differences in the nature of the substituents in the 5 and 6 position in the benzene ring. In the vitamins K there is a second benzene ring, in ubiquinone two methoxy groups, in tocopherylquinone and plastoquinone two methyl groups.

The side chain is fully saturated in α-tocopherylquinone, the double bond of the phytyl residue having water added across it. The single double bond of phytol is present in vitamin K_1, while the vitamins K_2 and ubiquinones have one double bond for each 5-carbon isoprene unit. There is a species difference in the length of the side chain in these compounds. Vitamin K_2 with a side chain containing twenty carbon atoms has been isolated[1] from animal tissue after feeding 2-methyl-1,4-naphthoquinone, while compounds with thirty, thirty-five and forty-five carbon atoms have been detected in bacteria.[2–5] Ubiquinone-50, with fifty carbon atoms or ten isoprene units in the side chain (Q_{10} in the alternative nomenclature) is found in most animal tissues[6,7] (the rat appears to be an exception with forty-five carbon atoms[8,9]) and ubiquinones-30, -35 and -45 have been isolated from yeast[10,11] and ubiquinone-40 from Azotobacter.[11] Plastoquinone[12–14] has forty-five atoms.

It should, perhaps, be emphasized at this stage that the close chemical resemblance between these groups of compounds is not reflected in close biogenetic interrelationships. It is hardly necessary to mention that the symptoms of deficiency of vitamin E in an animal are very different from those of vitamin K. Whether ubiquinone or its aromatic nucleus is a vitamin is not yet known. Several laboratories[15–18] have reported that the animal does not synthesize it from vitamin E. Also Morton[19] has shown no change in ubiquinone content in vitamin E or K deficiency (see, however, Green et al.[20]). In fact, there appears to be a closer biogenetic relationship between ubiquinone and the chemically unrelated vitamin A.[6,21]

QUINONES, QUINOLS, CHROMANOLS AND CHROMENOLS

In general, compounds of the type shown in Figure 1 can exist in four forms: two at the oxidation level of the quinone, two at the oxidation level of the quinol (Fig. 2). We shall now review what is known of the existence of these four forms in the four series—ubiquinone, vitamin E, vitamin K and plastoquinone—i.e., which of the possible sixteen compounds have been described.

Quinones. All four are known.

Chromenols. The chromenol of ubiquinone was recently characterized by Morton and his colleagues in Liverpool.[22] Solanochromene is the corresponding compound related to plastoquinone.[23,24] It is probable that compounds analogous to ubichromenol exist also in the

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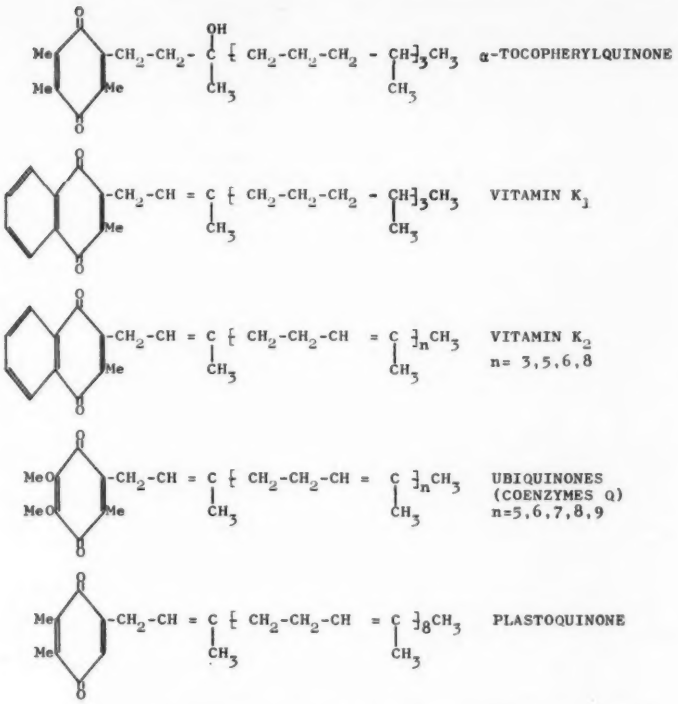

FIG. 1. Chemical structures of naturally occurring quinones.

vitamin K and tocopherylquinone series, but they have not yet been described.

Quinols. All the *p*-quinones described easily yield the quinol on the addition of reducing agents; e.g., $NaBH_4$ in ethanolic solution.

Chromanols. Vitamin E is a chromanol. The chromanol of vitamin K_1 (naphthotocopherol) was prepared in 1940 by Tishler and Fieser[25] by reduction of vitamin K_1 under acid conditions. In 1956, Bouman and I[26,27] came across the chromanol of ubiquinone (or a derivative of it), without at first recognizing it, when we treated the nonsaponifiable matter of a respiratory-chain preparation with ascorbic acid in HCl. We shall have more to say about this later. In the laboratory, we called this compound ubitocopherol (by analogy with naphthotocopherol), but recently proposed the name ubichromanol, in order to conform with Morton's terminology of ubichromenol. Ubichromanol has been recently prepared and thoroughly characterized by Folkers.[28]

Treatment of tocopherylquinone with ascorbic acid in HCl gives α-tocopherol in about 90 per cent yield.[27] With ubiquinone, the

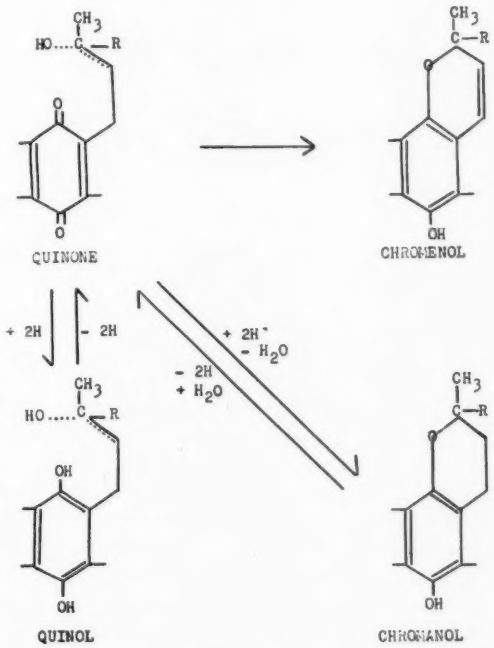

FIG. 2. Interrelationships between quinones, chromenols, quinols and chromanols.

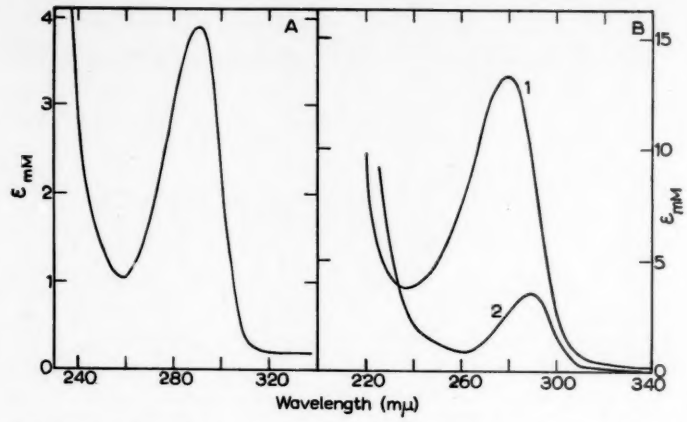

FIG. 3. A; absorption spectrum of ubichromanol obtained by the action of HCl-ascorbic acid on ubiquinone, followed by purification on alumina. B; absorption spectrum of $AuCl_3$-oxidation product of ubichromanol, before (curve 1) and after (curve 2) addition of KBH_4. (From: SLATER, E. C., RUDNEY, H., BOUMAN, J. and LINKS, J. *Biochim. et biophys. acta.* In press.[16])

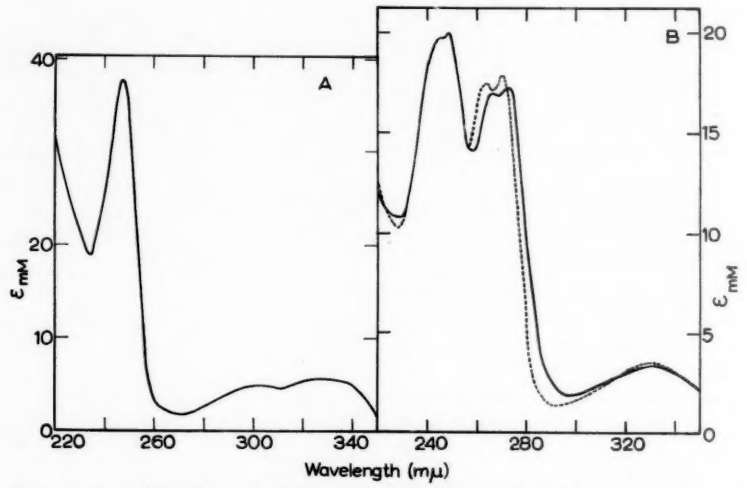

FIG. 4. A; absorption spectrum of naphthotocopherol obtained by action of HCl-ascorbic acid on vitamin K_1, followed by purification on alumina. B;..... absorption spectrum of vitamin K_1; ——— absorption spectrum of $AuCl_3$-oxidation product of naphthotocopherol. (From: SLATER, E. C., RUDNEY, H., BOUMAN, J. and LINKS, J. *Biochim. et biophys. acta*, 47:497, 1961.[16])

yield of ubichromanol is about 30 per cent. Ubiquinol is also formed, but is rapidly oxidized to the quinone during subsequent handling particularly if this includes adsorption on alumina.[16] The ubichromanol, on the other hand, is relatively stable to atmospheric oxygen. Figure 3 shows the absorption spectrum of ubichromanol (Fig. 3A), derived by the acid reduction of ubiquinone, the quinone obtained by oxidation of ubichromanol with $AuCl_3$ (curve 1, Fig. 3B), and the quinol obtained by reduction of this quinone with KBH_4 (curve 2, Fig. 3B). The maximum of ubichromanol is at 292 mμ, exactly the same as that of α-tocopherol. Ubiquinol is at 290 mμ. The quinone and quinol whose spectra are shown in Figure 3B are not identical with ubiquinone and ubiquinol, since the absorption maximum

of the quinone is at 279 mμ instead of 275 mμ, the maximum of ubiquinone. This is almost certainly due to the fact that, like α-tocopherylquinone, this product contains a γ-OH instead of the β, γ double bond present in ubiquinone. Similarly, the maxima of trimethylphytylbenzoquinone are 2 mμ further to the blue than those of α-tocopherylquinone.[16]

Vitamin K_1 chromanol can be prepared in the same way. Figure 4 shows its spectrum and that of its $AuCl_3$-oxidation product. The dotted line shows the spectrum of vitamin K_1. Again, the peaks of the $AuCl_3$-oxidation product of the chromanol are displaced a few millimicrons to the red with respect to the original quinone.

The chromanol of plastoquinone has not yet been described.

WHICH OF THESE COMPOUNDS ARE FOUND IN NATURE?

Table I shows which of these compounds has been found in nature. In the *vitamin E series*, only the chromanol has been shown unequivocally to be present, and in the *vitamin K series* only the quinone. In the *ubiquinone series* the quinol was discovered by Green and his colleagues[7] in their first work on Q_{275}. The chromenol as well as the quinone was isolated from human kidney and other sources as long ago as 1953 by Morton and his colleagues.[29] There is still some uncertainty whether ubichromenol exists in nature. Links,[30] in our laboratory, found that ubiquinone can be converted to ubichromenol by adsorption on basic alumina. The degree of conversion is less after acid washing, or after "weakening" the alumina by the addition of water, but even under those conditions it is not negligible; e.g., 6 per cent of the ubiquinone disappeared after twenty-four hours on acid-washed alumina containing 2 per cent water. Since Morton used adsorption on alumina to isolate ubichromenol, and found small quantities of ubichromenol in the presence of large amounts of ubiquinone, Links' observations raised some doubt as to the existence of ubichromenol in nature. Links' findings have been confirmed by Isler[31] and by Folkers.[32] Green[33] believes that the conversion to ubichromenol occurred during Links' experiments not during adsorption on the column, but during elution with acid. This is not correct. Links (unpublished) found that ubichromenol could be eluted not only with acid, but with organic solvents, while Folkers[32] has shown that acid does not convert ubiquinone to ubichromenol.

TABLE I

Presence of Quinones, Chromenols, Quinols and Chromanols in Nature

Compound	Vitamin E	Vitamin K	Ubiquinone	Plastoquinone
Quinone	−	+	+	+
Chromenol	−	−	?	?
Quinol	−	−	+	+
Chromanol	+	−	−	−

NOTE: (+) denotes that the compound has been isolated from natural materials; (−) denotes that its presence has not yet been established; (?) denotes that it has been isolated, but it is not yet certain that it is not an artifact of isolation.

Independent evidence for the idea that ubichromenol might be an artifact has come from Draper,[34] who found that ubiquinone was converted to ubichromenol when it was refluxed in ethanolic KOH. Most significantly, no ubichromenol was found in tissues when a method which avoided saponification was used.

On the other hand, Morton reported to the Ciba Foundation Conference on "Quinones in Electron Transport" that the ubichromenol which he isolated was optically active, which would suggest that it is a natural product.

Ubichromanol has not been found in nature.

WHICH OF THESE COMPOUNDS ARE PRESENT IN MITOCHONDRIA?

The fact that quinones can be reversibly reduced to quinols naturally raises the possibility that these compounds are involved as catalysts in intracellular respiration, a process known to be confined to the mitochondria. Martius[35] was the first who laid stress on this possibility. The next question is, then, which of these compounds is present in mitochondria?

Tocopherol. We showed some time ago that mitochondrial preparations contained α-tocopherol.[26,27] At first, we thought that they also contained tocopherylquinone, but after the demonstration by the Wisconsin

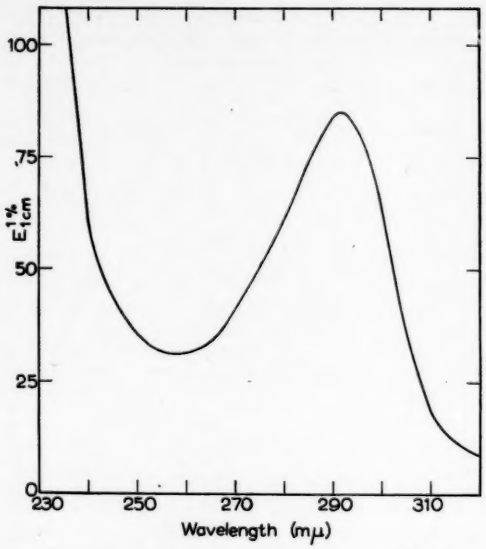

FIG. 5. Absorption spectrum of α-tocopherol isolated from ox-heart-muscle preparation. (From: SLATER, E. C., RUDNEY, H., BOUMAN, J. and LINKS, J. *Biochim. et biophys. acta*, 47: 497, 1961.[16])

group[7] of coenzyme Q or ubiquinone, we found that we had mistakenly identified ubichromanol as tocopherol in the products of acid reduction.[15,16,36,37]

Tocopherol isolated from heart-muscle preparation by a method not involving acid reduction has been identified by its similar behaviour to authentic α-tocopherol in chromatography on $ZnCO_3$-impregnated paper[38] and on alumina (the elution pattern being identical to that of the radioactivity derived from added ^{14}C-labeled α-tocopherol), by its ultraviolet absorption spectrum (Fig. 5) and by the absorption spectrum characteristic of α-tocopherylquinone obtained by oxidizing with $AuCl_3$.

When the extracted lipid was first treated in the presence of pyrogallol with ascorbic acid in HCl before saponification and subsequent chromatography on alumina, two peaks of reducing activity were found in the eluates. The first was α-tocopherol, and the second ubichromanol (or a closely related compound) derived from the ubiquinone present in the heart-muscle preparation. Both solutions had very similar absorption spectra, but were distinguished by the spectra of the $AuCl_3$-oxidation products.[15,16,37]

Experiments with added ^{14}C-labeled α-tocopherol showed that little, if any, α-tocopherylquinone is present in the heart-muscle preparation. A small amount of the labeled α-tocopherol was added before the chromatography, and the specific activities were measured of different portions of the one peak obtained when the nonsaponifiable matter was not subjected to acid reduction, and of the two peaks obtained with acid reduction. The radioactivity ran together with the reducing activity in the first peak, confirming that this was indeed α-tocopherol. The specific activity was not significantly lowered by acid reduction. If α-tocopherylquinone had been present in appreciable amounts, it would have yielded α-tocopherol on acid reduction, thereby diluting the radioactive α-tocopherol. Calculations show that less than 0.1 μM α-tocopherylquinone/gm. protein could have been present in the heart-muscle preparation, which contained 0.9 μM α-tocopherol/gm. protein. By direct chromatography by the method of Diplock et al.,[39] we have been unable to show any α-tocopherylquinone in heart-muscle preparation under conditions in which 0.02 μM/gm. protein would have been detected. Green has reported 0.05 μM α-tocopherylquinone/gm. protein by this method.[40]

Possible Function of α-Tocopherol. Since this heart-muscle preparation is quite free from substrates, it contains all its oxidizing catalysts in the oxidized form. The finding then that it contains α-tocopherol but little, if any, α-tocopherylquinone leads to the inescapable conclusion that the transformations α-tocopherol $\rightleftharpoons$ α-tocopherylquinone, or α-tocopherylquinol $\rightleftharpoons$ α-tocopherylquinone play no role in the respiratory chain.

However, there still remains to be considered the possibility that α-tocopherol is required for operation of the respiratory chain, without itself undergoing oxido-reduction, in the same way as the -SH group of coenzyme A is required for the oxidation of pyruvate and α-ketoglutarate without being oxidized to -S-S. In recent years, the main evidence for the function of α-tocopherol in respiratory-enzyme systems has come from Nason's[41] studies on the reactivation of iso-octane-treated preparations by the addition of α-tocopherol. These studies

have been very valuable in directing attention to the possible function of lipids in the respiratory chain. We were able to confirm Nason's experimental findings, but expressed some doubts as to whether added tocopherol really worked by replacing α-tocopherol extracted from the preparation by the iso-octane.[42] We suggested that the experiments could also be explained by the reverse phenomenon, namely that iso-octane was adsorbed on the particles thereby causing inhibition of the enzyme system, while the α-tocopherol removed the iso-octane. Reports from a number of laboratories[43–46] leave little doubt now that this explanation is correct. For a time, there remained two observations which appeared to be more easily explained on the basis of a catalytic role for α-tocopherol, *viz.* that *d*-tocopherol was more active than *dl*- in reactivating an iso-octane-treated preparation,[42,43] and that α-tocopherol also increases the activity of a preparation which is not treated with iso-octane.[41] Berne[47] has carefully reinvestigated the first point and found, in disagreement with a previous report from our laboratory and also from another,[43] that there is in fact no difference between the two forms. The second point has been investigated by Pollard and Bieri,[48] who found that this action of tocopherol could be explained by the reversal by α-tocopherol of the effect of an inhibitor, probably a lipid peroxide. Pollard and Bieri[49] have also reported that the DPNH-cytochrome *c* reductase activity of a particulate preparation from the hearts of vitamin E deficient chicks was normal, even though no trace of the vitamin could be detected. Of course, if the chicken heart can beat without any vitamin E, the vitamin is not necessary for intracellular respiration, and it is scarcely necessary to measure the activity of a respiratory enzyme.

We are left, then, with no evidence that α-tocopherol has any function in intracellular respiration. Yet, it is found in mitochondria. In fact, all the α-tocopherol in heart muscle (and also all the ubiquinone) is in the mitochondria.[16] It also appears significant that Nason[41] has shown that α-tocopherol is concentrated together with the DPNH-cytochrome *c* reductase during purification of this enzyme from bovine heart muscle. The concentration of α-tocopherol (0.4 μM/gm. protein) which we find in horse-heart-muscle preparation (0.8 μM/gm. protein in beef heart) is of the same order as that of known components of the respiratory chain. This concentration, which represents only about 0.03 per cent of the lipid, appears to be rather small for a structural role, and suggests rather a catalytic function. We keep the possibility in mind that α-tocopherol functions as a fat-soluble phenol which we should like to have as an intermediate of oxidative phosphorylation, but we have no real evidence. In fact, added α-tocopherol like other phenols is distinctly uncoupling. However, it is possible that α-tocopherol dissolved in the lipid phase of the mitochondria behaves differently from added α-tocopherol.

TABLE II
Concentrations of Various Components in Horse Heart-Muscle Preparation

Component	(μM/gm.) Protein
α-Tocopherol	0.4
Ubiquinone	9.4
Cytochrome ($c + c_1$)	0.8
Diaphorase (lipoate dehydrogenase)	0.1

Table II shows the mean concentrations of ubiquinone and α-tocopherol in the horse-heart-muscle preparation in comparison with those of cytochrome ($c + c_1$) and of a typical flavoprotein. Ox preparation has twice as much α-tocopherol and only one-third the amount of ubiquinone.

Function of Ubiquinone. Although it has come on the scene as a possible component of mitochondrial respiration much later than vitamin K or tocopherol, ubiquinone has rightly now taken the centre of the stage. The amount of ubiquinone in our horse-heart-muscle preparation is much greater than that of α-tocopherol, being somewhat greater than the concentration of the pyridine nucleotides in intact mitochondria. There is a good reason to believe that the quinone $\rightleftharpoons$ quinol transformation functions in the respiratory chain.[50] The recent measurements by Chance and Redfearn[51] of the rate of oxidation and reduction of the endogenous ubiquinone is impressive evidence that it is a component of the chain,

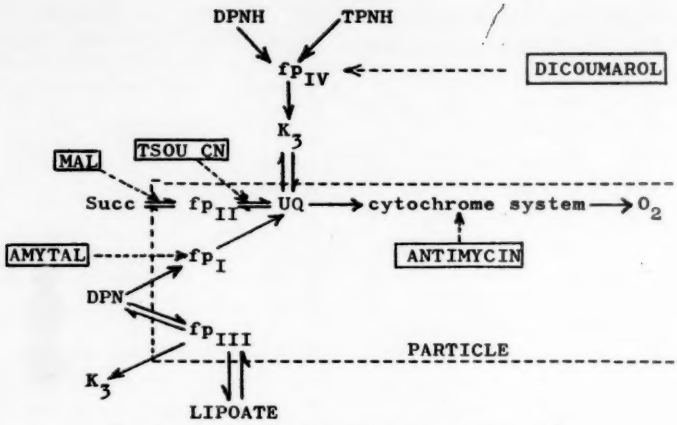

FIG. 6. Place of ubiquinone in the respiratory chain.

although Redfearn, no doubt wisely, is still cautious in his interpretation.[52]

This is not the place to discuss our own studies on the function of ubiquinone in the respiratory chain, which are essentially supplementary to the more important work of the groups led by Green,[53] Morton[54] and Chance.[51] The place in the respiratory chain which we assign to ubiquinone is shown in Figure 6, which is a simplified version of that recently presented.[55] Components present in the particles are shown within the dotted lines. The full arrows show the direction taken by two H atoms or electrons from substrate on to oxygen. The dotted arrows show the point of action of inhibitors, on which this scheme is based. Three of the flavoproteins present in the preparation are shown: succinic dehydrogenase (fp_{II}), the DPNH-oxidizing enzyme (fp_I) and lipoate dehydrogenase (fp_{III}) which is identical with the old diaphorase of Straub. We place ubiquinone between the flavoproteins and the cytochrome system. Menadione (vitamin K_3) can enter the chain as an hydrogen acceptor at two points. The one through ubiquinone is the only important pathway for the oxidation of reduced vitamin K_3, which we studied some time ago,[57] before the discovery of ubiquinone in mitochondria.

Also included in this scheme is the dicumarol-sensitive flavoprotein which catalyses the oxidation of both DPNH and TPNH by quinones. It is variously known as menadione reductase,[59] phylloquinone reductase,[59] vitamin K reductase[60] and DT diaphorase.[61] This flavoprotein reacts with a number of acceptors, including both benzoquinones and naphthoquinones.[60,61] There is some conflict of evidence whether it reacts with vitamin K, or ubiquinone. Martius[60] and Ernster[61] find no activity, but Wosilait[62] does. Ernster[63] has shown that this enzyme together with menadione and mitochondria provides a system for the oxidation of DPNH or TPNH by an antimycin-sensitive pathway. No physiologic function can yet be ascribed to this enzyme, because menadione has not been shown to be present in the soluble fraction of cells. It is by means certain that this enzyme has a function in intracellular respiration.

Possible Function of Vitamin K in the Respiratory Chain. This raises the question of the possible role of vitamin K in the respiratory chain, which was postulated some time ago by Martius.[35] The reason why we have always been rather reluctant to accept this proposition is that, according to a chick assay test carried out for us in Professor Dam's laboratory,[26] the heart-muscle preparation contains less than 0.01 μM vitamin K/gm. protein; i.e., less than one-thousandth the concentration of ubiquinone. Although it can be said, of course, that the mitochondrial preparation contained the vitamin K but that this was lost in making the nonphosphorylating heart-muscle preparation, the fact remained that *all* the α-tocopherol and ubiquinone are retained in this preparation.[16] Lester and Crane[11] were also unable to find any vitamin K in beef heart lipids. Edwin et al.[40] have re-

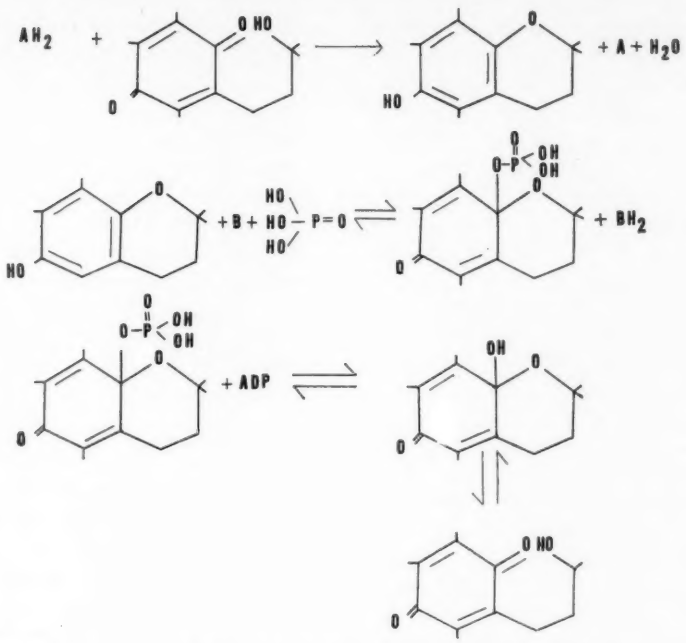

FIG. 7. A possible function of quinones in oxidative phosphorylation.

ported very small amounts of vitamin K_1 in a heart-muscle preparation. Against these reports stands an indirect determination by Martius[64] that rat-liver mitochondria and especially heart sarcosomes contained appreciable amounts of ^{14}C-labeled vitamin K_1 after feeding ^{14}C-labeled menadione.

My view is that more direct evidence for the presence of vitamin K in animal mitochondria is necessary before we can accept that this vitamin is concerned in oxidative phosphorylation in animals. There is more reason to suppose that it has this function in bacteria, particularly in those which do not contain ubiquinone.

POSSIBLE ROLE OF QUINONES IN OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation accompanying the oxidation of menadiol by mitochondria has been demonstrated.[57] In these experiments, menadiol is used as a substrate for the respiratory chain and the observation that phosphorylation accompanies the oxidation in no way proves that the quinone or quinols have any direct role in oxidative phosphorylation. For example, it is quite possible that the phosphorylation steps with either succinate or menadiol as substrate lie entirely in the cytochrome system and that those portions of the chain lying between succinate or menadiol and cytochrome are not directly associated with phosphorylation reactions.

Ubiquinone and ubiquinol do, however, offer attractive possibilities for paper biochemistry with respect to oxidative (or photosynthetic) phosphorylation. Most of the various reaction schemes which have been proposed are equally applicable to vitamin K, tocopherylquinone, ubiquinone or plastoquinone.

Between 1954 and 1958 many authors[65–69] suggested the participation of a quinol phosphate as an intermediate in oxidative phosphorylation. Wessels[65] suggested that oxidation of the quinol phosphate would give rise to a high energy phosphate compound at the oxidation level of the quinone. The phosphate group in this compound could then be transferred to ADP in a separate reaction.

The other authors[66–69] who have supported the role of a quinol phosphate have suggested that the oxidation of the quinol to the quinone is accompanied by the simultaneous transfer

of the phosphate group to ADP. Model experiments of Wieland[67] and Clark et al.[69] have shown that such a reaction is feasible.

The weakness of these theories is that they do not explain how the quinol phosphate is formed in the first place.

Another type of theory involving quinones in oxidative phosphorylation, proposed at the Fourth International Congress of Biochemistry,[37] is depicted in Figure 7. This scheme was originally proposed for tocopherylquinone, but can be applied almost unchanged to vitamin K, or ubiquinone (which have the β,γ double bond in the side chain instead of the -OH). According to this mechanism, the quinone is reduced with cyclization to the chromanol by the reduced member of the respiratory chain, designated AH_2 (i.e., analogous to treatment of these compounds with ascorbic acid in HCl). Oxidation by B, in the presence of phosphate,* forms a phosphorylated compound at the oxidation level of the quinone which can transfer its phosphate to ATP. The sum of these reactions is the oxidation of AH_2 by B coupled with the phosphorylation of ADP by H_3PO_4.

Dallam[70] has proposed a somewhat similar mechanism. The first reaction is identical. The second is a phosphorylytic cleavage of the chromanol to form a quinol phosphate, which is oxidized with simultaneous transfer of phosphate to ADP, as in the theories of Todd and others.[66–69]

The latest theory of Todd[71] involves exactly the same intermediate phosphorylated compound, but it is formed by direct action of phosphate on the quinone. The quinone phosphate is then reduced to a quinol phosphate, which is oxidized with simultaneous synthesis of ATP.[66–69]

It must be emphasized that, however attractive quinones are for suggesting mechanisms of oxidative phosphorylation, the direct participation of either the quinone or the quinol in the phosphorylation reaction has not yet been proved. Very recently, however, Brodie[72] has reported the isolation of a phosphorylated derivative of the chromanol of vitamin K_1, after incubation of substrate, vitamin K, and particles of Mycobacterium phlei. We are very interested in the reported presence of ubichromenol. If what Morton and U. Green have isolated is not an artifact arising from ubiquinone during the isolation procedure, it seems possible that it is derived from some intermediate form of ubiquinone in oxidative phosphorylation. We are currently exploring this possibility.

* An alternative version[37] of this theory involves "I," the hypothetical link between oxidation and phosphorylation, as the participant in this reaction instead of phosphate. A subsequent phosphorolysis substitutes the phosphate group for I, yielding the same phosphorylated intermediate as in the mechanism shown in Figure 7.

SUMMARY

A survey is given of the chemical properties of four biochemically important groups of compounds: the vitamins E, the vitamins K, the ubiquinones and plastiquinones. All four groups are known in the form of the *p*-benzoquinone. The isomeric chromenol has been described in the ubiquinone and plastoquinone series. All four *p*-quinones can be reduced to the quinol, or, an acid solution, to the chromanol (the chromanol of plastoquinone has not yet been described). Only a few of these compounds are known with certainty to be present in nature—the quinone in the vitamin K, ubiquinone and plastoquinone series, the quinol in the ubiquinone and the chromanol in the vitamin E series.

The possible biochemical function of α-tocopherol and ubiquinone, both of which are localized in the mitochondria, is discussed. There is no direct evidence that α-tocopherol has any function in intracellular respiration, but the possibility remains open. There is good reason to believe that ubiquinone is a component of the respiratory chain operating between the flavoproteins and the cytochrome system. The possible function of vitamin K in intracellular respiration is also discussed.

Possible reaction schemes for the participation of quinones and related compounds in oxidative phosphorylation are given.

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The Inhibition of Ascorbic Acid Synthesis by the Process of Lipid Peroxidation in Vitamin E Deficiency

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FIFTEEN YEARS have passed since the proposal of the hypothesis that the action of tocopherol was in some way connected with its antioxidant properties. This hypothesis is supported by the fact that peroxides are increased in preparations from vitamin E deficient animals and by observations that other antioxidants with unrelated chemical structures can substitute for tocopherol in preventing the symptoms and serious consequences resulting from the deprivation of this vitamin. Only recently, this hypothesis has been considerably strengthened through the work of Draper and his colleagues,[1] who have demonstrated survival of three successive generations of rats in whose diet tocopherol had been substituted by a synthetic antioxidant. Implicit in the antioxidant hypothesis is the idea that particular oxidation products of certain metabolites are noxious or toxic to vital cell processes and their production must not be allowed to develop. Of the various possible metabolites, lipids were selected from the beginning as the responsible group; the most likely reason for this selection being the lipid peroxidation has been a problem of long-standing for the food technologist.

The antioxidant hypothesis was confronted with a rather cold reception from biochemists, perhaps because it is too indefinite. The precise nature and mechanism of action of the toxin whose production is prevented by antioxidants has not been worked out. It is possible to suggest more than one mechanism for the mode of action of the toxic factor, but many investigators have considered with favor the idea that these oxidation products can be enzyme inhibitors. As a matter of fact, several papers have been published listing enzymes which, are inhibited by the addition of different peroxides.[2–4] Unfortunately, the fact is that not one of these enzymes has been found to be inhibited or diminished in tissues of vitamin E deficient animals. Table 1 is a list of enzymes which, for one reason or another, we have tested in our laboratories in attempts to detect enzymic differences between the tocopherol-sufficient and tocopherol-deficient animals. Some of the activities listed involve systems of enzymes, and consequently the inhibition of only one of them to a level at which it would become the rate-limiting reaction of the entire process would then place the entire sequence on a list of possibly peroxide-susceptible enzymes. In addition to this list, we have investigated and found the *in vivo* turnover of phosphates to be accelerated in the vitamin E deficient rabbit.[5] If we extend this list to at least some of the findings of other laboratories, we find that total oxygen consumption[6] and amino acid[7] and nucleic acid[8] turnovers have been reported to be accelerated rather than diminished. Several of the enzymes in Table 1 appear to be inhibited, but we have failed to find the conditions necessary so that all animals placed under these conditions will consistently show those differences before visible cell deterioration occurs.

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TABLE I

Effect of Tocopherol Deficiency on Various Enzymes of the Rat and Rabbit

Enzyme	Animal	No. of Experiments	Vitamin E Sufficient	Vitamin E Deficient	Units
Metalloenzymes or Enzymes Requiring Metal Cofactors					
Arginase	Rat	7	.010	.011	mg. N
Muscle dipeptidase	Rat	5	105	129	O.D.* 570 mμ
Liver dipeptidase	Rat	3	171	120	O.D. 570 mμ
Intestinal alkaline phosphatase	Rat	2	26.3	22.9	μM $HPO_4^=$
Liver alkaline phosphatase	Rat	2	.697	.403	μM $HPO_4^=$
Catalase	Rat	6	5.36	4.50	mEq. $NaBO_3$ consumed.
Thiol Group, Containing Enzymes					
Sulphatase C	Rat	6	62.3	57.3	O.D. 500 mμ
Glycerophosphate-dehydrogenase	Rat	4	.649	.646	O.D. change/min., 340 mμ
D-amino acid oxidase	Rat	24	.433	.360	μM O_2/hour
DPNH-cytochrome *c* reductase	Rat	7	.464	.435	O.D. change/min., 340 mμ
Enzymes Concerned with Phosphate Metabolism					
Oxidative phosphorylation	Rabbit	6	2.88	2.34	P/O
Creatine phosphokinase	Rabbit	39	.513	.370	μM $HPO_4^=$
Phosphoglucomutase	Rabbit	38	.383	.341	μM G-6-P
Glycolysis	Rabbit	22	5.68	6.36	μM CO_2/gm. tissue/30 min.

* O.D. = Optical density in this and other tables.

TABLE II

Rate of Ascorbic Acid Synthesis and Destruction by Liver Preparations from Animals on Different Diets

Animal	Name of Diet*	No. Pairs of Animals	Supplement to Diet	Ascorbic Acid Synthesis and Destruction (μM/gm. wet tissue/two hours†)
				Synthesis
Rat	BD + A and D	14	None	0.52 ± 0.14
			Vitamin E	1.62 ± 0.25
	BD + 1.5% C.L.O. + A and D	13	None	0.29 ± 0.07
			Vitamin E	1.37 ± 0.19
	BD + 3.0% C.L.O.	9	None	0.26 ± 0.08
			Vitamin E	1.00 ± 0.12
	BD + 1.5% C.L.O. + A, D and C	11	None	0.18 ± 0.07
			Vitamin E	2.38 ± 0.19
Rabbit	BD + 3.0% C.L.O.	8	None	0.31 ± 0.10
			Vitamin E	1.47 ± 0.26
				Destruction
Rat	BD + 1.5% C.L.O. + A and D	4	None	1.37 ± 0.10
			Vitamin E	1.25 ± 0.12

* BD = basal diet; C.L.O. = cod liver oil; A, D and C are vitamins A, D and C supplements.
† Mean ± standard error.

INHIBITION OF ASCORBIC ACID SYNTHESIS IN VITAMIN E DEFICIENCY

The synthesis *in vitro* of ascorbic acid from glucuronic acid is the only reaction which has been found invariably inhibited in vitamin E deficient animals.[9] Table II shows that this effect is observed with different low tocopherol diets. The first diet is very similar to that devised by Mason and Harris to be used for

TABLE III

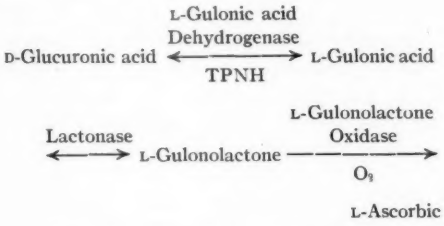
Reactions of the Ascorbic Acid Synthesis Sequence in Preparations from Vitamin E Deficient Rats and Their Control Group

Dietary Group	Enzyme Preparation	Addition to Test System	No. of Experiments	Production or Consumption of TPNH, Oxygen or Ascorbate (μM produced or consumed/hour/gm. liver)	TBA Reaction* ΔO.D. 535 mμ
				TPNH†	
E deficient	Dialyzed supernatant	...	8	3.74	0
E sufficient	Dialyzed supernatant	...	8	3.96	0
				Oxygen‡	
E deficient	Supernatant	...	3	8.8	...
E sufficient	Supernatant	...	3	3.6	...
				Ascorbate§	
E deficient	Microsomes	...	7	1.9	.484
E deficient	Microsomes	Tocopherol	2	3.3	.057
E deficient	Microsomes	Co^{++}	2	4.1	.016
E deficient	Microsomes	EDTA	2	4.1	.024
E sufficient	Microsomes	...	7	3.8	.021
E sufficient	Microsomes	Tocopherol	2	3.5	.018

* Method of Ottolenghi.[13]
† Reduced triphosphopyridine nucleotide, determined spectrophotometrically at 340 mμ.
‡ Determined manometrically.
§ Method of Roe and Keuther (1943).[9]

inducing experimental sterility in the rat; it does not produce muscular dystrophy in the rabbit. The other diets are increasingly richer in cod liver oil. The one containing 3 per cent cod liver oil produces muscular dystrophy in the rabbit regularly in three or four weeks The effect of these diets on the synthesis of ascorbic acid is identical.

The following scheme illustrates the sequence of enzymic steps in ascorbic acid synthesis from glucuronic acid.[10]



The scheme may become slightly more complicated in the future, but we can feel confident that by and large the two first activities are in the cell sap while the third activity is present in the microsomes. Table III shows that only the microsomal activity (L-gulonolactone oxidase) is inhibited in extracts obtained from vitamin E deficient rats or rabbits. The first enzymic step in the sequence was also tested in the same extracts (gulonic acid dehydrogenase) but was not affected, or may even be increased. In addition, the lactonase involved in this process was determined (not shown in the table) but was also found unaffected.

Investigation of the affected enzymic step revealed that the inhibition could be abolished by the addition of a number of substances to the enzymic assay system (Table IV). At first, the diverse nature of the activators gave no clue to the manner in which they were functioning.[11,12] The list would be somewhat bewildering except for the other column in this table which shows the results of the thiobarbituric acid (TBA) reaction. The TBA reaction, as carried out,[13] is a specific method to determine malonaldehyde, which is a product consistently obtained in the process of lipid peroxidation. These data are interpreted to mean that any agent which will stop lipid peroxidation will prevent the inhibition of L-gulonolactone oxidase.

Figure 1 shows that the pigment obtained from the reaction of thiobarbituric acid and the peroxidation product formed during the incubation of the enzyme preparation behaves the same chromatographically as the pigment formed by the reaction of thiobarbituric acid

TABLE IV

Effects of Various Agents on the Synthesis of Ascorbic Acid and on the Production of Thiobarbituric Acid-Reacting Material by Liver Preparations of Vitamin E Deficient Rats and The Control Group

Additions to Test System (final concentration)	Supplement to Basal Diet	Ascorbic Acid Synthesis			Thiobarbituric Acid Reaction		
		No. of Experiments	(μM/gm. liver/2 hours)		No. of Experiments	ΔO.D. 535 Mμ	
			No Addition	With Addition		No Addition	With Addition
Co^{++} 5×10^{-4} M	None	9	0.25	1.71	5	0.257	0.028
	Vitamin E	7	1.47	2.00	3	0.005	0.011
Mn^{++} 5×10^{-4} M	None	9	0.28	1.22	4	0.375	0.028
	Vitamin E	7	1.39	1.68	4	0.014	0.000
Ce^{+++} 5×10^{-4} M	None	6	0.36	1.20	4	0.487	0.060
	Vitamin E	2	1.31	1.02	3	0.000	0.000
Fe^{++} 5×10^{-4} M	None	5	0.43	0.08	6	0.351	0.535
	Vitamin E	3	1.16	0.52	4	0.000	0.000
Fe^{+++} 5×10^{-4} M	None	4	0.46	0.29	4	0.351	0.535
	Vitamin E	2	1.22	1.07	2	0.000	0.000
$SeO_3^{=}$ 5×10^{-4} M	None	2	0.29	0.21	2	0.481	0.458
	Vitamin E	2	1.33	0.72	2	0.010	0.028
EDTA 6×10^{-5} M	None	13	0.21	1.36	6	0.258	0.025
	Vitamin E	7	1.53	1.22	4	0.019	0.020
Santoquin®* 2 mg./ml.	None	2	0.31	1.00	2	0.281	0.000
	Vitamin E	2	1.19	0.56	2	0.000	0.000
DPPD† 2 mg./ml.	None	1	0.47	1.02	2	0.487	0.000
	Vitamin E	1	0.93	0.96	1	0.003	0.002
α-Tocopherol	None	1	0.480	0.712	1	0.524	0.012
15 mg./gm. wet liver	Vitamin E	...	...	...	...	...	...
Vitamin A alc.	None	1	0.480	0.652	1	0.524	0.010
15 mg./gm. wet liver	Vitamin E	1	0.750	...	1	0.000	...
Vitamin K_1	None	1	0.480	0.651	1	0.524	0.064
15 mg./gm. wet liver	Vitamin E	1	0.750	0.618	1	0.000	0.008
Vitamin D	None	1	0.480	0.390	1	0.524	0.350
15 mg./gm. wet liver	Vitamin E	1	0.750	0.610	1	0.000	0.000

* 1 2 dihydro-6-ethoxy-2,2,4 trimethyl quinoline.
† N,N'-diphenyl-p-phenylenediamine.

with authentic malonaldehyde. In addition to the chromatographic identity, the ultraviolet spectra of both pigments are identical at any pH. As further proof that the TBA reactant was malonaldehyde, the chromogen was isolated and oxidized with hydrogen peroxide with the result that malonic acid was obtained. There is, therefore, practically no doubt that the substance is malonaldehyde, and assuming that this substance is an actual indicator of lipid peroxidation, this process occurs in our enzymic system, but only when the enzyme, gulonolactone oxidase, is actively functioning.

TABLE V

Malonaldehyde Production and Ascorbic Acid Synthesis by the 40 and 90 Microsomal Fractions

Fractions	Additions (fraction) plus substance)	Ascorbic Acid Synthesized (μM/2 hours)	Malonaldehyde Produced (O.D. 535 mμ)
40	...	0.34	0.000
90	...	0.05	0.717
40 + 90	...	0.17	1.100
40 + heated 90	...	0.09	0.830
Heated 40 + heated 90	...	0.00	0.018
40	90 + lipid	0.07	0.747
40	90 lipid + tocopherol	0.41	0.005

STUDIES WITH SOLUBILIZED L-GULONOLACTONE OXIDASE

A method for the solubilization and fractionation of L-gulonolactone oxidase was developed in our laboratory by Drs. R. E. Trucco and A. E. Kitabchi,[14] and has provided a much needed tool for the further analysis of this phenomenon. Tabie v shows that in the above mentioned fractionation, a separation of the active enzymic component from the lipid inhibitor or inhibitor precursor was obtained.

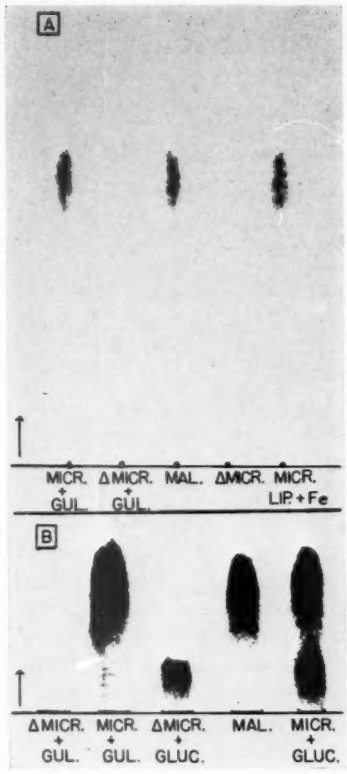


FIG. 1. Chromatography of TBA complexes of chromogens produced by liver microsomes of vitamin E deficient rats. In A, the solvent was pyridine-butanol-water, 3:2:1.5 and in B, 85 per cent ethanol. MICR. = microsomes; Δ MICR. = microsomes heated in boiling water bath for two minutes before incubation; MICR. LIP. = chloroform-extracted lipid fraction of microsomes; GUL. = gulonolactone; GLUC. = glucuronolactone; and MAL. = synthetically prepared, 2 times crystallized malonaldehyde-TBA pigment. Pigments were extracted from the TBA reaction mixture (previously extracted with ether) with HCl-isoamyl alcohol (1:1), immediately applied to paper and air dried. After descending irrigation, the dried papers were photographed by contact printing in the panel light copier and Autostat developing process of Apeco (Dallas, Texas). Spots with less than 0.5 μg. of malonaldehyde are visualized by this procedure.

The fraction designated as 40 is one which was obtained from the solubilized enzyme preparation by precipitation with ammonium sulfate to 40 per cent saturation. On increasing the ammonium sulfate concentration to 90 per cent saturation, another fraction is obtained which is called the "90" fraction. It is apparent from the table that the enzyme and the inhibitor can be separated, and that the inhibition is not due primarily to the lack of a cofactor but rather to the presence of an inhibitor occurring in the original preparation. The solubility characteristics of this inhibitor indicate that it is a lipid.

TABLE VI
Effect of Malonaldehyde on L-Gulonolactone Oxidase Activity*

Malonaldehyde Added (γ/ml.)	Ascorbic Acid Synthesized (μM/hour)	TBA Reaction (O.D. 532 mμ)
0	0.55	.130
0.1	0.54	.185
1.0	0.49	.632

* System: Enzymes, 0.25 ml.; phosphate buffer, .15M, pH 7.5, 0.75 ml.; incubated one hour at 37°c.

These preparations also made it possible to determine whether or not the inhibitor is initially present in the enzymic system or if it is formed during the reaction. In earlier studies, the first indication that the inhibitor was formed during the reaction was observed in a study of the behavior of malonaldehyde-forming process in the presence of tocopherol, Co^{++}, Mn^{++} and EDTA.* Figure 2 shows that the formation of malonaldehyde is stopped by these reactivating agents but that the aldehyde is not destroyed. However, this was insufficient proof and the study was repeated after the separation of the enzyme and the inhibitor (or inhibitor precursor) was achieved. An experiment was devised in which the same preparation, with and without the potential inhibitor, was compared. The results showed that the respective reactions progress at the same rate during the first thirty minutes of incubation at which time the reaction stops rather abruptly in the presence of the inhibitor. The inhibitor appears to be produced from a lipid precursor present in the enzyme preparation. The ratio of activities of both reactions should be an expression of the amount of inhibitor present at any moment and was found to follow an exponential slope similar to that seen in a free radical-type reaction.

At this point it should be made clear that malonaldehyde is a product derived in the

* EDTA = ethylene diamine tetra-acetic acid.

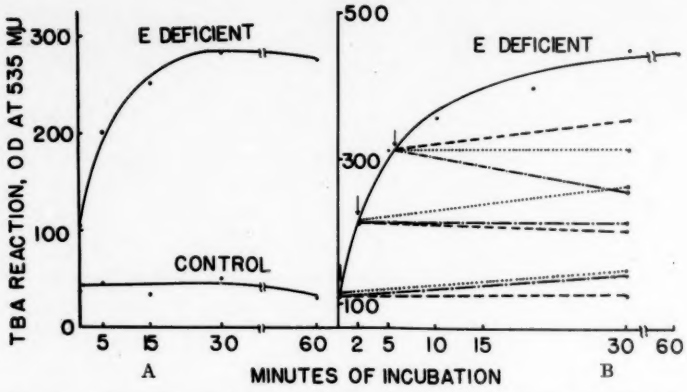
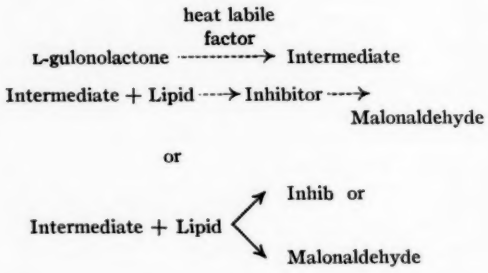


FIG. 2. A, peroxide production by liver preparations of vitamin E deficient and control rats. B, effects of addition of Co^{++} *in vitro* – – –; EDTA · · ·; and tocopherol - . - . - on the production of peroxides by liver preparations of vitamin E deficient rats. Arrows at zero, two and five minutes indicate the times at which additions were made. Thiobarbituric acid reaction is shown as O.D. at 535 $m\mu \times 10^3$.

formation and breakdown of the lipid inhibitor, but it does not show in itself inhibitory properties (Table VI). The following is a simple scheme of what we believe to be the sequence of events resulting in the inhibition of gulonolactone oxidase:



STUDIES ON THE MECHANISM OF THE INHIBITION

We are confronted at the present time with the problem of determining the chemical basis of the susceptibility of this enzyme to the toxic peroxidation process. One hypothesis considered was that peroxides formed from the lipid will attack thiol groups in the enzymes. Since the enzyme has not been purified, and very little is known of its nature,[15,16] it seemed at the time that the best method for attacking this problem was to study the reaction of other enzymes with sensitive sulfhydryl groups to the peroxidation process. The following enzymes were studied: D-amino acid oxidase, microsomal reduced diphosphopyridine nucleotide (DPNH) oxidase, sulfatase C, and glycerophosphate dehydrogenase. As stated before, none of these were inhibited in extracts of the vitamin E deficient tissue in which the peroxidation process was occurring. In another set of experiments, the results obtained were also not in agreement with this hypothesis. Table VII shows that when either gulonolactone oxidase or D-amino acid oxidase are inhibited by peroxides produced by aging linolenic acid in the presence of oxygen and then added to the tissue extract, the enzymes are inhibited and the inhibition can be reversed by addition of reduced glutathione. But when the inhibitor is produced from the, as yet, unidentified tissue lipid during the incubation, the addition of reduced glutathione is ineffective as an activator of gulonolactone oxidase.

THE CHEMICAL NATURE OF THE INHIBITOR

We are at present interested in examining the nature and, if possible, the biologic significance of this inhibitor. At the present time, there is neither evidence in favor of nor against the possibility that this inhibitor plays a role *in vivo* comparable to that which it plays *in vitro*. We were unable to demonstrate an increased malonaldehyde level in the tissues of vitamin E deficient animals. Our results are in discrepancy with those published by other authors, but we believe this is due to a differ-

TABLE VII

Effect of Glutathione on Gulonolactone Oxidase and D-Amino Acid Oxidase

Diet Fed	Additions to Incubation System (final concentration)	No. of Experiments	Gulonolactone Oxidase Activity (μM ascorbic acid/hour)	No. of Experiments	D-Amino Acid Oxidase Activity (μM O_2/hour)
		In Vitamin E Deficiency			
E sufficient	...	3	0.55	5	.62
E sufficient	3.2×10^{-3} M GSH	3	0.51	0	...
E deficient	...	3	0.10	5	.62
E deficient	3.2×10^{-3} M GSH	3	0.12	0	...
		On the Inhibition by Oxidized Linolenic Acid			
Rockland laboratory	...	3	0.29	0	...
Rockland laboratory	$+4.4 \times 10^{-4}$ M unoxidized linolenic acid	3	0.30	0	...
Rockland laboratory	...	2	0.56	1	2.77
Rockland laboratory	$+4.4 \times 10^{-4}$ M oxidized linolenic acid	2	0.12	1	0.89
Rockland laboratory	$+4.4 \times 10^{-4}$ M oxidized linolenic acid + 3.2×10^{-3} M GSH	2	0.36	1	2.78

ence in the technic used in the determination of malonaldehyde *in vivo*. Our determinations have been carried out in tissues homogenized in trichloroacetic acid to avoid the *in vitro* formation of malonaldehyde that occurs in buffers. Table VIII demonstrates that when determinations are done in this way, no difference is found between vitamin E sufficient and deficient animals. These results, however, do not rule out the existence of an increased peroxidation *in vivo* in vitamin E deficient animals. Meehan, in our laboratories, injected 2.9 mg. of malonaldehyde into rats and found that after ten minutes only 56 and 34 per cent, respectively, could be recovered in vitamin E sufficient and deficient animals (Table IX). This amount, 2.9 mg., is two or three times greater than the calculated amount produced by 200 gm. of liver *in vitro* in one hour. Unfortunately, these results leave the important question of whether or not there is an increased production of peroxides *in vivo* an unsolved question.

A new line of investigation was started in our laboratories recently. If it is correct that the type of inhibition observed is not produced by the formation of peroxides from all of the different unsaturated fatty acids, it is probable that the inhibition is attributable

TABLE VIII

Concentration of Malonaldehyde in Various Tissues from Animals with Vitamin E Deficiency

Tissue	No. of Analyses	Vitamin E Sufficient (μg.)	Vitamin E Deficient (μg.)
Liver	6	.23	.21
Testis	6	.14	.17
Muscle	6	.12	.16
Brain	6	.22	.23
Kidney	4	.27	.25
Spleen	4	.28	.37
Adrenals	2	.14	.15

TABLE IX

Fate of Malonaldehyde (2.9 mg.) Injected Intraperitoneally into Rats

Dietary Group	Per cent of Malonaldehyde Recovered		
	Ten Minutes after Injection (%)	Twenty-Four Hours after Injection (%)	
	Carcass	Carcass	Excreta
E sufficient	56	0	10.8
E deficient	34	0	11.1

TABLE X
Effect of Tissue Lipid Fractions on L-Gulonolactone Oxidase Activity

Source of Lipid	L-Gulonolactone Oxidase Activity (O.D. 540 mμ)*			
	Lipid Fraction Added†			
	None	PL	NF	FA
E deficient liver	251	100	238	160
E sufficient liver	390	275	350	325
E deficient muscle	176	110	...	179
E sufficient muscle	390	227	282	298

* For method see reference 9. † PL = phospholipid fraction; NF = neutral fats fraction; FA = fatty acids fraction.

to an effect produced by some specific precursor. In either case, a quantitative study of the inhibitor precursor should be useful for an evaluation of the effect. This has been done in a rough manner by studying the total chloroform-methanol extract of several organs from vitamin E deprived animals. Using equal amounts (by weight) of the extracted materials, it was found that their inhibitory potency decreased in the following order: muscle, brain, liver, and testes. None of the inhibitions with these lipid extracts were very impressive, however, probably due to the presence of some anti-inhibitor substance which may well be tocopherol itself. The total chloroform-methanol extracts were then fractionated. The results of testing these fractions for inhibitory properties are shown in Table X. It can be seen that the inhibition is due almost entirely to the phospholipid fraction. The neutral fat and the free fatty acids fractions have little or no inhibitory activity.

COMMENTS

There can be little doubt that in the tissue preparations of vitamin E deficient animals conditions develop which can lead to the inhibition of L-gulonolactone oxidase as a result of the peroxidation of cellular lipid which occurs simultaneously with the activity of the enzyme. These conditions do not seem to be the same as those created in a system where the autoxidation of unsaturated fatty acids had occurred as a consequence of a previous exposure to O_2, even though this system also inhibits L-gulonolactone oxidase. It is noteworthy that while the peroxides formed by autoxidation of these acids appear to be inhibitors of many enzymes, only one enzyme thus far has been found to be inhibited by the process of peroxidation characteristic of vitamin E deficient animals.

It is not known at the present time whether the factor responsible for the inhibition is absolutely transient or might somehow be stabilized. Up to now, the inhibition by the tissue lipid has been observed only after a thirty minute incubation of the enzyme under study, and no method has been devised by which the inhibitor can be transferred from one system to another without altering the inhibitor in the process. An inhibitor of a transient nature would naturally lead one to consider that it might be a free radical.

The relevance of the processes described in this presentation to an explanation of the symptoms of vitamin E deficiency has not been assessed yet. The changes in oxygen tension and the destruction of the organization which occurs in the transformation of a cell into a homogenate should make one cautious in drawing conclusions about a process in which, conceivably, so much depends on the oxygen tension and cell organization.

SUMMARY

The mechanism of inhibition of gulonolactone oxidase in liver homogenates from vitamin E deficient rats and rabbits appears to involve the reaction of either an intermediate formed in the enzymic conversion of gulonolactone to ascorbic acid or ascorbic acid itself with a lipid component in the system to produce an inhibitor. The inhibitor or a by-product of its production eventually degradates to yield detectable amounts of malonaldehyde. The action of tocopherol in this sequence would be to prevent the interaction of the postulated intermediate and some component of the tissue lipids.

Although gulonolactone oxidase is inhibited in liver homogenates from vitamin E deficient rats and rabbits, and the inhibition is attributed to lipid peroxidation in the enzyme system, there is, as yet, no direct evidence that a similar phenomenon takes place *in vivo*. The data show that the usual method for detecting lipid peroxidation *in vivo* (as defined in this report)

would probably give negative results even if such was occurring.

ACKNOWLEDGMENT

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DISCUSSION

Dr. F. A. Kummerow (*Urbana, Illinois*): The intravenous injection of as little as 10 mg. of the hydroperoxide of methyl linoleate, emulsified in 1 ml. serum, into chicks kept on a diet which contained 10 per cent corn oil (Table I) induced cerebellar disorders such as ataxia, tremors and retropulsive movement one to five hours after the injection. No cerebellar disorders were noted after injection in birds kept on the diet which had been supplemented with 8 mg. per cent α-tocopherol. The injection of 10 mg. of reduced hydroperoxide, methyl 12-oxo-*cis*-9-octadecenoate, or fresh methyl linoleate in 1 ml. of serum caused no cerebellar disorders. Thus, of the oxidized fatty acids tested, only lipoperoxide initiated symptoms of cerebellar disorders. Pathologic examination of tissue of the cerebellum indicated that the injection of methyl linoleate hydroperoxide caused considerable cerebellar edema.

The presence of the mixed tocopherols in corn oil might have partially prevented the destruction of the cerebellum, since 8 mg. per cent of additional α-tocopherol completely prevented the cerebellar disorders caused by the injection of methyl linoleate hydroperoxide. That the α-tocopherol naturally present in corn oil did influence the development of cerebellar disorders was shown in chicks kept on diets which contained corn oil stripped of vitamin E (Table II). The rate of incidence depended on the level of stripped corn oil; a high fat, high protein diet resulted in the

TABLE I

Effect of Intravenous Injection on Cerebellar Disorders

Lipid Injection	No Supplement	Tocopherol (8 mg. %)
Methyl linoleate hydroperoxide	5/8	0/8
Reduced methyl linoleate hydroperoxide	0/4	0/4
Methyl 12-oxo-*cis*-9-octadecenoate	0/4	0/4
Methyl linoleate	0/4	0/4

TABLE II

Effects of Composition of Diets on Chick Nutritional Encephalomalacia and on the Cerebellar Disorders Caused by the Injection of Methyl Linoleate Hydroperoxide*

Treatment No.	Soy (ADM) Protein† (%)	Stripped Corn Oil (%)	Ascorbic Acid (mg. %)	Inositol (mg. %)	Feed Intake 7 days (gm.)	Weight Gain 7 days (gm.)	No. Chicks with Encephalomalacia	Effect of Intravenous Injection of Methyl Linoleate Hydroperoxide in Survivors			
								Total No. of Chicks	No. of Chicks with Cerebellar Disorders	Chicks Showing Hemorrhage and Necrosis	
										No.	Percentage‡
1	35.30	2	0	0	146	96	0/12	6	4	1	25
2	35.30	10	0	0	142	96	5/12	6	6 (2)§	6 (2)	100
3	35.30	20	0	0	140	59	10/12	2	2	2	100
4	11.77	2	0	0	144	52	0/12	6	1	0	0
5	11.77	10	0	0	129	45	0/12	6	5	1	20
6	11.77	20	0	0	129	46	2/12	6	4	2	50
7	35.30	10	75	0	136	96	2/6	4	4 (2)	0	0
8	35.30	10	0	30	141	82	4/6	2	2 (2)	2 (2)	100
9	35.30	10	75	30	129	89	0/6	6	6 (2)	1	17
10	11.77	10	75	0	129	51	0/6	6	5	0	0
11	11.77	10	0	30	129	47	0/6	6	6 (1)	0	0
12	11.77	10	75	30	133	49	0/6	6	5	0	0

* Added to the high and low protein diet, respectively was 0.30 or 0.10 per cent glycine. Supplements to basal diet were made at the expense of glucose on weight basis.

† Manufactured by Archer-Daniels-Midland Company.

‡ Out of number which showed cerebellar disorders.

§ Number in parenthesis indicates shock-like symptoms developed in chicks.

highest incidence of encephalomalacia. Although the presence of either ascorbic acid or inositol alone was not effective in preventing a high incidence, the presence of both ascorbic acid and inositol and a low protein diet depressed the incidence of encephalomalacia.

When the chicks which had not developed symptoms of encephalomalacia were injected with emulsions of 10 mg. methyl linoleate hydroperoxide, all of the chicks which had been kept on diets high in protein and fat showed cerebellar ataxia and various degrees of degeneration, necrosis and hemorrhage of the cerebellar tissue after injection. Again the rate of incidence was lower on a low protein diet.

We noted that unless chicks showed positive symptoms of cerebellar disorders, no noticeable cerebellar lesions were present. It would seem, therefore, that diets which caused a high incidence of nutritional encephalomalacia also caused severe destruction of the cerebellar tissue after injection of methyl linoleate hyroperoxide into chicks which had not developed symptoms after one week on a vitamin E deficient diet.

A Possible Site of Action for Vitamin E in Intermediary Metabolism

KLAUS SCHWARZ, M.D.*

AS A COROLLARY to Dr. Caputto's presentation, I would like to discuss briefly the crucial points of some work carried out in the Section on Experimental Liver Diseases with the aim to identify the active site of tocopherol in enzyme systems. This work has been in progress since 1952 in conjunction with our systematic attempts to elucidate the causal chain of events leading from dietary deficiency and metabolic defects on the molecular level to liver necrosis and death. It was discovered early[1] that liver slices from rats on vitamin E deficient diets are incapable of maintaining normal oxygen consumption in the Warburg respirometer for more than thirty to sixty minutes (Fig. 1). The phenomenon, respiratory decline, is characteristic of the latent phase of the disease which precedes liver necrosis by ten to fourteen days.[2] During this phase gross or even microscopic changes are not detectable but serious damage to mitochondria and microsomes is evident from electron microscope pictures.[3] Respiratory decline is indicative of a specific function of tocopherol in the maintenance of normal energy metabolism. The impairment is prevented by feeding vitamin E; it disappears within ten minutes after injection of physiologic amounts of α-tocopherol into the portal vein or into peripheral vessels.

It would not be feasible to discuss these studies here in detail. Some of the results are mentioned elsewhere in this monograph.[4] A thorough analysis of the effects of thirteen different antioxidants has shown that a few of them are quite potent as dietary supplements, and also following intraportal application, while the majority of "run of the mill" antioxidants, especially those which are used commercially for the stabilization of fats, are inactive. When added to the slice medium directly in form of emulsions, tocopherol is completely inactive, while those antioxidants which are effective after injection are also effective *in vitro*. The observations indicated to us several years ago (1956) that tocopherol is converted in intermediary metabolism into an active form.[2]

We have searched for this active form by testing of various tocopherol derivatives.[5] Thus far, the only substance derived from tocopherol and effective in the prevention of respiratory failure in our *in vitro* experiments with liver slices is the metabolite described by Simon et al.[6,7] (Fig. 2). The material prevents respiratory decline when added at relatively low amounts. Fifty per cent prevention will occur with roughly 6 μg. of the Simon metabolite per 3 cc. of medium and 100 mg. of slices (Table 1). Parenthetically, with 100 μg. or more of the metabolite, a pronounced stimulation of respiratory activity has been observed. From a comparison of the biopotency of various substances effective in our system, we feel that the Simon metabolite, which is excreted in the urine as the glucuronide, is not the active form of vitamin E. But it seems to be related to it, possibly as a breakdown product of the active compound.

Approximately one and a half years ago it was detected by our group* that phosphate-buffered media permit the observation of respiratory decline in homogenates of vitamin

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* Experiments carried out by L. M. Corwin.

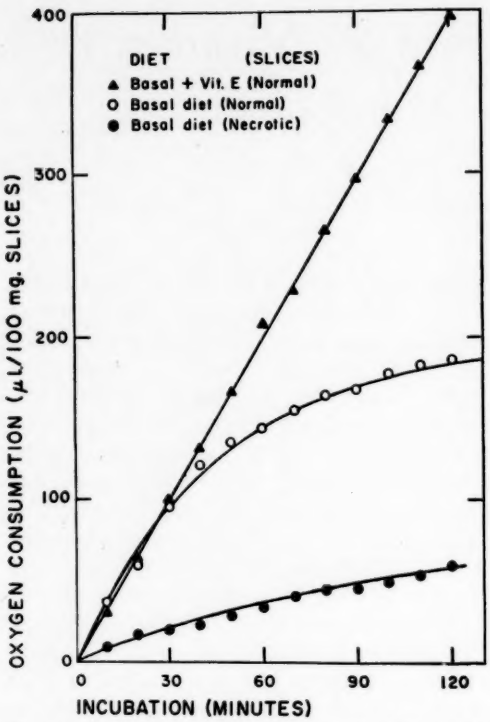

Fig. 1. Respiratory decline of liver slices from rats during the latent phase of dietary necrotic liver degeneration.[2]

E deficient livers.[8] This system has been used for a new approach to the problem at hand. It permits analysis of variables which could not be tested in liver slices. Variation of substrates, especially of members of the Krebs cycle, showed that respiratory decline is a general phenomenon; it occurs also with glutamate, β hydroxybutyrate, etc. With certain substrates tocopherol dependent decline is more rapid and more pronounced than with others. For instance, pyruvate-malate and α-ketoglutarate presented a large degree of imbalance while citrate and succinate were affected least. Supplementation of unchanged α-tocopherol to the medium was without effect, similar to the results obtained by addition of tocopherol directly to liver slices. However, when the vitamin was rehomogenized with the liver homogenate, protection was achieved. The tocopherol metabolite of Simon et al., and also menadione, methylene blue, and especially DPPD were found to be potent agents in the prevention of the metabolic lesion.

Preceding studies had shown that deficient mitochondria alone were metabolically intact and did not respond to conditions of the *in vitro* experiment with loss of respiratory activity,[9] in contrast to the whole homogenate. The only defect detectable in the mitochondria of E deficient animals was seen in succinate utilization in the presence of DPN. In this case respiration started out relatively fast but came to a standstill in a short period of time. The phenomenon was shown to be correlated to an increased accumulation of oxalacetic acid. The latter is a potent negative feedback inhibitor of succinic dehydrogenase. The exact reason for the enhanced accumulation, or rather the apparent decreased removal of oxalacetic acid from the system has not been clarified.

The results with mitochondria alone, when compared to those with liver homogenates or liver slices, showed clearly that an interaction between various components of the liver cell was involved in the elicitation of respiratory failure. By combining various cell fractions after differential centrifugation it was shown that the microsomal portion greatly accelerated the respiratory failure when combined with the mitochrondria while the supernatant had little or no effect.[10] The mechanism of this interaction is not entirely understood at present. Thus far, attempts to extract inhibitory agents from microsomal preparations have led only to partial success. As in Dr. Caputto's system, a trace element seems to be involved.

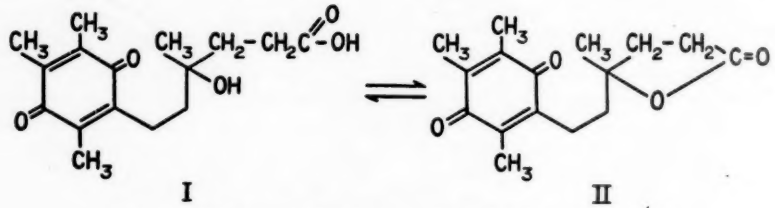

Fig. 2. Urinary tocopherol metabolite isolated by Simon et al.[7]

TABLE I
In Vitro Effect of Tocopherol Derivatives on Respiratory Decline
(*Liver Slices*)[5]

Supplement	Dose in Flask	No. of Experiments	Respiratory Decline (%)		Prevention (%)
			Control	With Supplement	
Tocopherol-metabolite (free acid, quinone form)	6.25	5	73	39	50 ± 20.6
	25.00	4	71	13	83 ± 4.4
	100.00	5	70	4	92 ± 5.3
DL-α-Tocopherol	200.00	5	72	66	(8 ± 3.6)
d-α-Tocopheryl polyethylene glycol-1000 succinate	1000.00	5	75	72	(5 ± 4.2)
DL-α-Tocopherylhydroquinone	200.00	5	71	77	(−12 ± 12.7)
DL-α-Tocopherylquinone	200.00	4	72	74	(−3 ± 6.4)
DL-α-"Tocopheroxide" (acetal of quinone)	200.00	4	67	77	(−19 ± 11.7)

TABLE II
Prevention of Decline of α-Ketoglutarate Oxidation by GSH and BAL
(*Liver Homogenate*)*

Addition (μM)	No. of Rats	Time Interval (min.; μatoms 0/50 mg.)		
		0–30′	60–90′	Decline (%)†
...	9	10.5 ± 0.5	3.2 ± 0.4	69 ± 3
0.3 GSH	3	10.0 ± 0.8	4.4 ± 0.7	55 ± 4
1.0 GSH	5	9.5 ± 0.5	9.7 ± 0.4	↑3 ± 7‡
0.1 BAL	3	9.3 ± 0.7	3.1 ± 0.8	65 ± 12
0.3 BAL	5	8.5 ± 0.3	9.1 ± 0.3	↑7 ± 6‡

* Experiment by L. M. Corwin. † Decline (%) = 100 × (0–30′) − (60–90′)/0–30′. ‡ ↑ = per cent increase.

Following the discovery by McLean that EDTA prevents respiratory decline in liver slices,[11] it was shown that EDTA and other complexing agents prevented the respiratory breakdown in our homogenate system as well.* The specific nature of the element which might be involved has not been established conclusively, but it seems likely that iron is the effective ingredient.

It is interesting to note that the metabolic impairment in liver slices is also prevented by reduced glutathione and by BAL. Relatively small amounts of these agents are likewise effective in our homogenate system. BAL is more active than the glutathione-SH (Table II). In separate studies it was shown by inhibition analysis that the chain of electron carriers does not seem to be involved directly in respiratory decline. It was concluded that the systems which are primarily affected, most likely through attack and inactivation by a trace element, are those dealing immediately with the substrates; i.e., the various dehydrogenase systems. These are all sulfhydryl enzymes.

The various pieces of evidence conveyed here can be integrated to indicate that tocopherol may have a physiologic function in close relationship to thiol or dithiol groups of enzyme systems. The exact nature of this interaction on the molecular level remains to be clarified. From the fact that the vitamin itself is inactive, while the Simon metabolite is effective, one could conclude that not α-tocopherol but rather a conversion product of the latter participates in the metabolic reaction. It is noteworthy that the glutathione content of liver tissue in animals maintained on our basal diet is greatly reduced compared

* Experiments carried out by L. M. Corwin.

TABLE III

In Vitro Effect of Protecting Agents on Free Sulfhydryl Groups (*Liver Homogenate*)*

Addition (γ)	Decline (%) of α-KG Oxidation	μM-SH/50 mg.†
...	70 ± 5	0.62 ± 0.10
10 Tocopherol metabolite	↑ 12 ± 3↑	0.91 ± 0.06
10 Menadione	16 ± 4	1.00 ± 0.11
1 Methylene blue	↑ 24 ± 6‡	0.97 ± 0.10
1 DPPD	↑ 20 ± 7‡	1.14 ± 0.06

* Experiment by L. M. Corwin.

† The quantity of sulfhydryl groups at 0 time was 1.06 ± .06.

‡ ↑ = per cent increase.

TABLE IV

Prevention of Decline Due to Arsenite and Cd++ by α-Tocopherol (*α-Ketoglutarate and DPN*)*

Addition (μM)	Decline (%)	
	Without Tocopherol	With 20 γ Tocopherol
...	8 ± 8	↑ 23 ± 3↑
0.1 AsO_2	14 ± 10	↑ 19 ± 6↑
0.3 AsO_2	57 ± 6	7 ± 7
0.01 Cd++	48 ± 8	14 ± 5
0.02 Cd++	56 ± 10	↑ 7 ± 5↑

* Experiment by L. M. Corwin. The system used was a combination of mitochondria and supernatant fractions, omitting the nuclear and microsomal fractions. 10 γ tocopherol were homogenized with each fraction to total 20 γ per flask.

↑ ↑ = per cent increase.

to normal. It is also known that feeding of ample supplements of sulfur amino acids to animals on the Torula yeast diet delays the development of respiratory decline and of liver necrosis by ten to twelve days. Detailed studies of the interrelationship between sulfur amino acid supplementation and tocopherol requirement for protection against liver necrosis have revealed that sulfur amino acids decrease the requirement for α-tocopherol to approximately only one-tenth of that normally required.[13]

Determinations of free sulfhydryl groups in liver homogenates under our experimental conditions[12] have shown that the substances which actively prevent respiratory decline also prevent the disappearance of free sulfhydryl groups during the incubation (Table III). To a certain degree, maintenance of normal respiration and of free SH groups in the homogenate are correlated with each other. It is possible that this parallelism is of deeper significance. However, one must keep in mind that the simultaneous observation of two phenomena does not mean that one is etiologically the cause of the other.

If one takes α-ketoglutarate oxidase, a relatively well known enzyme system, as an example, it becomes evident that different types of sulfhydryl groups could be more or less specifically involved in the protective effect which is elicited by tocopherol after rehomogenization, by the tocopherol metabolite, but also by menadione and especially DPPD. The dehydrogenase itself contains vicinal dithiol groups in the form of thioctic acid at its active site. The α-ketoglutarate oxidase system also contains various other sulfhydryl groups. It is particularly sensitive to poisoning by reagents such as arsenite or cadmium. The mechanism of this inhibition and its prevention by BAL and other dithiol compounds has been analyzed by Sanadi and others.[14] They correlated the inhibition to the fact that the dehydrogenase contains the vicinal dithiol groups of thioctic acid. Inhibition is caused by reaction of the metals with the dithiol groups. In our laboratory it has been established by Corwin that the inhibitory effect of arsenite and cadmium on α-ketoglutarate oxidation of normal liver homogenates can readily be antagonized by those agents which are effective in the prevention of respiratory decline, specifically by rehomogenized tocopherol (Table IV).

The protective effect of the metabolically active form of tocopherol and of the other agents effective in our system could be interpreted in the following two ways:

1. The active compounds, in the oxidized form, i.e., quinones, could interact with sulfhydryl groups at the active sites by an oxidation-reduction reaction which forces the equilibrium towards the S-S form. Such a shift in the equilibrium would effectively eliminate the point of attack of the small amounts of inhibitory heavy metals which appear to be causing respiratory failure. The reduced form of the tocopherol derivative

could be reoxidized metabolically. It is very well known, indeed, that tocopherol itself and reduced tocopherol derivatives undergo oxidation by reacting with iron (III). Thus, one could conceive of the possibility that the hypothetical metabolite serves as an intermediate carrier for electron transfer from reduced mono- or dithiol sites of enzymes or cofactors to iron containing catalysts, for instance, members of the cytochrome chain, or to other iron containing enzyme sites.

2. An alternate possibility is given by the fact that quinonoid structures readily form reaction products with sulfhydryl groups by simple condensation. Such products are chemically well defined and known, for instance, for cystine and benzoquinone,[15] and for glutathione and menadione.[16] It seems possible that tocopherol metabolites of a quinonoid nature, as well as the other active substances mentioned, have a masking or shielding effect on labile SH groups by virtue of this mechanism. One can envision that such reaction products of quinones with sulfhydryl sites are the truly effective, electron transferring configurations. Their formation and stability would be impaired by heavy metals, on one hand, and enhanced by the active compounds, on the other hand.

It is hoped that further pursuit of the approach described here may lead to the identification of the metabolic function of tocopherol on the molecular level. It is my conviction that tocopherol, in its active form, has a distinct catalytic role in intermediary metabolism. I cannot conceive of tocopherol, a vitamin, simply as of a "policeman" trapping radicals and keeping oxygen molecules in line which stray out of line by accident. The antioxidant function may be strictly coincidental to the true metabolic function of the vitamin.

By way of conclusion, I would like to venture a thought which may sound like heresy to some. In the controversy about "specific metabolic function" vs. "antioxidant activity" we may be dealing with a classic case of a pseudoargument. We should not forget that in some specific instances tocopherol is not an anti- but a potent pro-oxidant. It is possible that peroxides are actually normal products in intermediary metabolism and that they fulfill a perfectly useful function in certain oxidative pathways. If lipid peroxides, for instance of essential fatty acids, would be such short lived, normal intermediates, then the active form of tocopherol could be the catalyst which metabolized them further. There is nothing I know which would preclude such possibilities except for the fact that we are not accustomed to think in these terms. The fact that we cannot demonstrate lipid peroxides in metabolizing systems does not mean very much. Scientists have tried in vain for thirty years to find acetate as a normal intermediate, and yet, as we all know, it is indeed a metabolite of utmost importance. At any rate, in the question of the metabolic functions of vitamin E, we have come to a point where a little bit of concrete, positive evidence may go a long way to clear up existing misinterpretations.[4]

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Metabolism of Alpha-Tocopherol and the Isolation of a Nontocopherol-Reducing Substance from Animal Tissues

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THE DESCRIPTION AND interpretation of the function of vitamin E in terms of a precise and well-defined biochemical reaction common to all tissues and cells still represents an unsolved problem in nutritional biochemistry.

Because of the susceptibility of vitamin E to oxidation and because of its replaceability by certain synthetic antioxidants, tocopherols are at present generally considered to be rather nonspecific physiologic antioxidants which protect the structural and metabolic integrity of intracellular units.[1–6]

Despite the attractiveness of the antioxidant theory, and even after the negative results reported on the proposed role of α-tocopherol as a cofactor of DNPH-cytochrome C-reductase,[7–10] it may still be possible to consider the participation of α-tocopherol or its active form as a cofactor in some specific reaction.[11–12]

One of the primary tasks in understanding the role of α-tocopherol either as a nonspecific antioxidant or as a cofactor would be the isolation, separation and identification of the metabolites of α-tocopherol. So far, nothing has been definitely known about the chemical transformations of α-tocopherol in the body. For this reason we have started an investigation on the metabolism of C^{14}-labeled α-tocopherol with the hope that once all metabolites have been identified one may be able to understand better the role and function of this vitamin.

It was recognized by Karrer et al.[13] and by John et al.[14] that α-tocopherol is readily oxidized to α-tocopherylquinone and that the latter can be reduced to the α-tocopherylhydroquinone in a neutral solution or reduced and recyclized to α-tocopherol in a strongly acidic solution. Boyer,[15] by the oxidation of α-tocopherol with ferric chloride in the presence of 2,2′-bipyridine in ethanolic solution, prepared a new oxidation product α-tocopheroxide which could readily be reduced to α-tocopherol or converted in an acid solution irreversibly to α-tocopherylquinone. At first Boyer believed that α-tocopheroxide, to which he had assigned an epoxy structure, represented an intermediate in the oxidation of α-tocopherol to α-tocopherylquinone. However, Martius and Eilingsfeld[16] have shown that α-tocopheroxide is a hemicetal of α-tocopherylquinone, formed only by oxidation of α-tocopherol in ethanolic solution. The possible chemical interconversions between α-tocopherol, α-tocopheroxide, α-tocopherylquinone and α-tocopherylhydroquinone are presented in classic scheme by Harrison et al.[17] (Fig. 1).

Polarographic studies by Smith et al.[18–19] and by Wachs[20] have shown that an electromotively-active bivalent reversible oxidation product is formed when α-tocopherol is oxidized at the dropping mercury electrode. Potentiometric titration[17] of α-tocopherol by auric chloride indicated an apparent oxidation-reduction potential of +0.187 volts, and the

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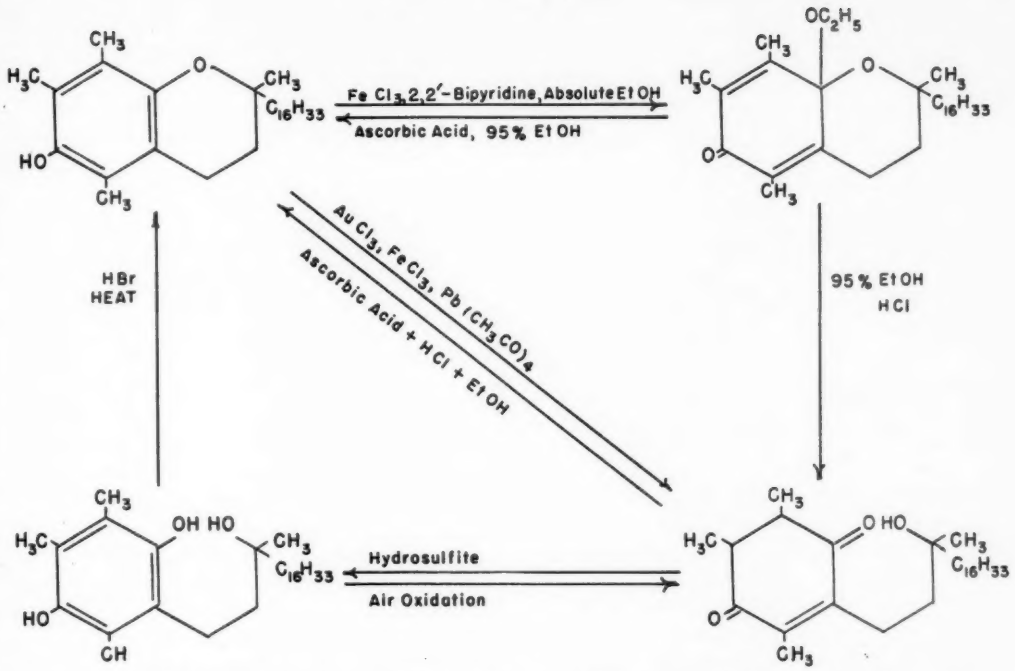

FIG. 1. The possible chemical interconversions between α-tocopherol, α-tocopheroxide, α-tocopherylquinone and α-tocopherylhydroquinone. (From: HARRISON, W. H., GANDER, J. E., BLAKELY, E. R. and BOYER, P. D. *Biochim. et biophys. acta*, 21: 150, 1956.[11])

only oxidation product formed during the titration was α-tocopherylquinone. Unfortunately, various mixtures of α-tocopherol and α-tocopherylquinone did not give any measurable potentials under the condition used for the titration, and therefore it was concluded that α-tocopherylquinone does not form an electromotively-active oxidation-reduction pair with α-tocopherol. A common intermediate, formed during the oxidation of α-tocopherol, has been proposed to account for all the chemical interconversions of known α-tocopherol compounds *in vitro*.[11,19,21] The bivalent oxidation of α-tocopherol results in the formation of a carbonium ion or tocopheryl cation (Fig. 2) which under nucleophilic attack by an ethoxy group or hydroxy group forms α-tocopheroxide or α-tocopherylquinone. This carbonium ion also represents the necessary intermediate for the reduction to α-tocopherol by ascorbic acid, stannous chloride or cysteine.

It has been generally thought that oxidation to α-tocopherylquinone represents the major step in the catabolic pathway of α-tocopherol and that further conversion to α-tocopherylhydroquinone also occurs.[1] Scudi and Buhs[22] and McCormick et al.[23] have claimed the presence of α-tocopherylquinone in human and animal plasma, Rosenkrantz et al.[24] have identified it in human stools on the basis of its

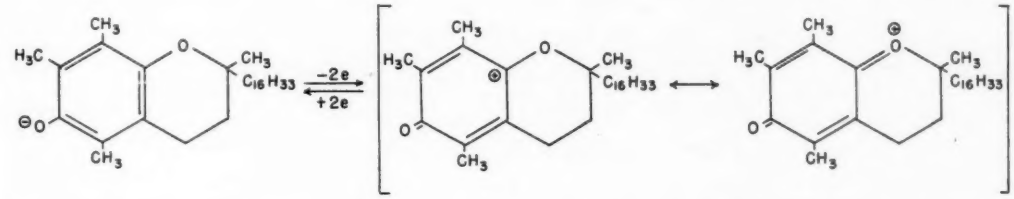

FIG. 2. The formation of α-tocopheroxide or α-tocopherylquinone from the bivalent oxidation of α-tocopherol.

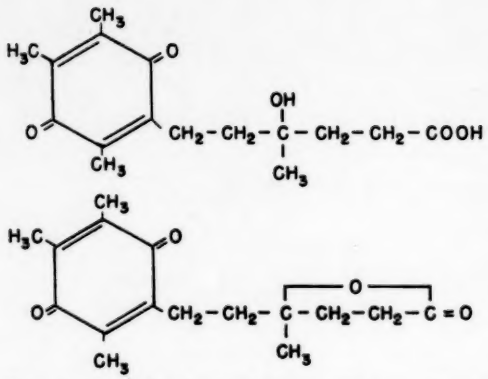

FIG. 3. Tocopherol metabolites. (From: SIMON, E. J., EISENGART, A., SUNDHEIM, L. and MILHORAT, A. T. *J. Biol. Chem.*, 221: 807, 1956.[30])

distribution constant and absorption spectra, and recently Diplock et al.[25] have reported spectroscopic and paper chromatographic evidence for its presence in horse heart mitochondria. It is interesting that Mervyn and Morton[26] found α-tocopherylquinone only in nephritic kidneys lacking α-tocopherol. On the other hand, Hines and Mattill,[27] Pollard and Bieri,[28] Slater,[11] and Draper and Alaupović[29] have been unable to demonstrate its presence in various animal tissues.

Simon et al.[30] isolated from rabbit and human urine and feces two α-tocopherol metabolites which were characterized as 2(3-hydroxy-3-methyl-5-carboxypentyl) - 3,5,6 - trimethylbenzoquinone and its γ-lactone (Fig. 3). These two compounds are the only well characterized α-tocopherol metabolites thus far discovered, and according to Simon et al. are probably formed by oxidation of α-tocopherol to α-tocopherylquinone and its subsequent reduction and conjugation with glucuronic acid, the terminal methyl group of the side chain then being oxidized to a carboxyl group which is conjugated with coenzyme A and degraded by β-oxidation until a six carbon chain is formed. The termination of β-oxidation can be explained by a nucleophilic attack by the hydroxy group on the acyl coenzyme A to form the acidic metabolite, and the quinones are formed during the isolation procedure.

Following the administration of radioactive α-tocopherol to rabbits, Martius and Costelli[31] found that, in addition to unchanged α-tocopherol, a compound could be isolated from the liver mitochondria which in its solubility properties resembled trimethylphytylbenzoquinone (Fig. 4) rather than α-tocopherylquinone. They suggested that this compound, which is reducible by acid reduction to α-tocopherol, is the functional form of vitamin E in the cells.

Sternberg and Pascoe-Dawson[32] made preliminary studies on the distribution of radioactivity in the different tissues of the rat after oral administration of C^{14}-α-tocopherol; however, these workers did not attempt to characterize any of the radioactive compounds.

In the work reported here we have administered radioactive tocopherol or tocopheryl succinate to rats and pigs. While we have been unable to detect α-tocopherylquinone or hydroquinone, or α-tocopheroxide or trimethylphytylbenzoquinone, or Simon's metabolites in the livers or tissues of these animals, we have found three metabolites, one of which (compound F) accounts for 28 to 50 per cent of the liver radioactivity.

ANIMALS AND MATERIALS

Crystalline d-α-tocopheryl-5-methyl-C^{14} succinate (obtained from Distillation Products Industries; specific activity 1.1 μc./mg.) was used for the preparation of free alcohol d-α-tocopherol-5-methyl-C^{14}, which was prepared either by saponification of the succinate ester with methanolic potassium hydroxide in the presence of pyrogallol or by reduction by lithium aluminum hydride. The free α-tocopherol was purified by chromatography on a silicic acid-Celite (2:1) column.

Normal male and female albino rats were

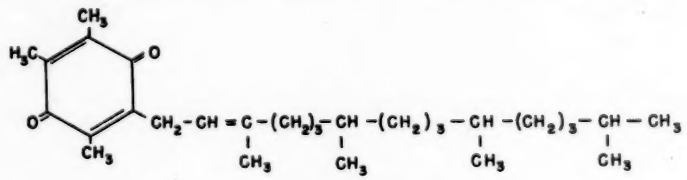

FIG. 4. Trimethylphytylbenzoquinone.

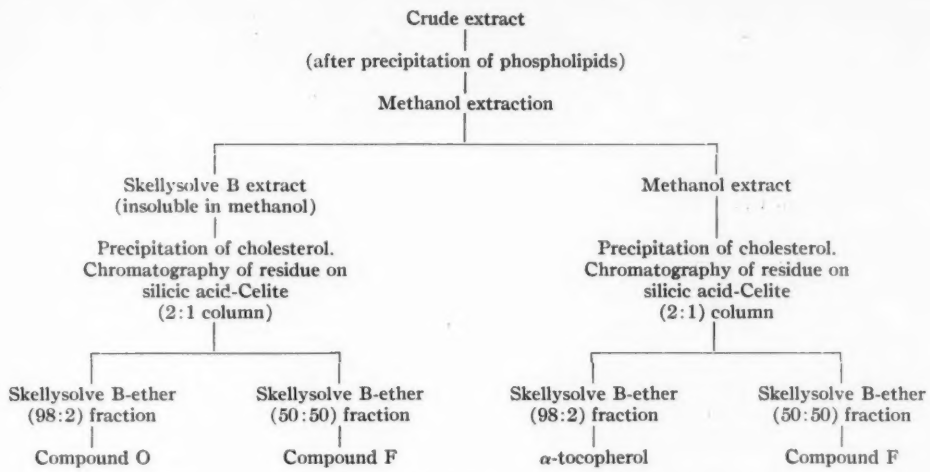

FIG. 5. Flowsheet for the isolation of α-tocopherol metabolites.

injected intraperitoneally with 1 to 2 mg. of d-α-tocopherol-5-methyl-C^{14} dissolved in 1 ml. of a mixture of 1 part of ethanol and 9 parts of 16 per cent aqueous Tween 80 solution. After forty-eight hours the animals were killed, the livers removed and immediately stored under ethanol, and the carcasses and excreta frozen.

In two experiments, normal pigs averaging 175 pounds were injected intraperitoneally with 17 to 20 mg. of labeled or unlabeled d-α-tocopherol in the same way as described for the rats. The animals were killed after forty-eight hours and the carcasses immediately frozen.

ISOLATION AND SEPARATION OF METABOLITES FROM LIVER

Individual pig livers and pooled rat livers were ground and extracted in the dark, in the presence of pyrogallol, with a mixture of 10 parts ethanol and 8.5 parts Skellysolve B for six hours in a mechanical shaker at room temperature. The extracted tissue was repeatedly washed with the same solvent and then with diethyl ether until the last traces of radioactive substances had been removed. The combined extracts were diluted with water, the Skellysolve layer was removed, and the aqueous ethanolic solution was re-extracted with Skellysolve B. The Skellysolve fractions were combined, the solvent was removed *in vacuo* under nitrogen, and the residue was dissolved in diethyl ether. All subsequent evaporations were also carried out *in vacuo* under nitrogen. The phospholipids were precipitated with acetone. The supernatant was evaporated to dryness and the residue was extracted with methanol. The methanol-insoluble residue was dissolved in Skellysolve B. The methanol and Skellysolve B extracts were chromatographed on silicic acid-Celite (2:1) columns which were developed with solvent mixtures consisting of Skellysolve B and increasing percentages of diethyl ether. The separation of unchanged α-tocopherol, and two metabolites, compound O and compound F, is shown in Figure 5.

Fractions containing compound O were purified by chromatography on silicic acid columns, and then by preparative paper chromatography, using the Eggitt and Ward[33] reverse-phase system; this was followed by chromatography on a secondary magnesium phosphate column[34,35] and precipitation in absolute ethanol at −70°c. (Fig. 6). Compound O was insoluble in ethanol at −70°c. (compound Oi) and showed a UV λ max at 283 mμ. (ethanol). Compound Os, which was soluble in ethanol at −70°c., showed a λ max at 257 mμ. ($E^{1\%}_{1cm}$ = 12.3 in isooctane).

Compound F was purified by chromatography on an alumina column and on a secondary magnesium phosphate column (Fig. 7). This oily substance showed an inflexion at 270 mμ. ($E^{1\%}_{1cm}$ = 20.1 in isooctane).

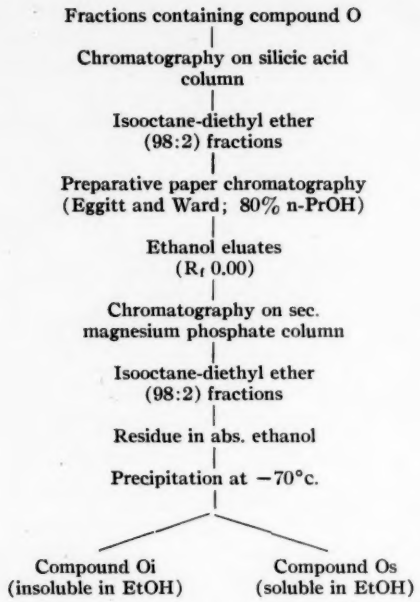

FIG. 6. Purification of compounds O.

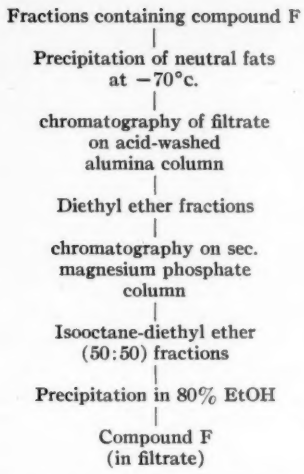

FIG. 7. Purification of compound F.

RESULTS

The amount of pure new metabolites isolated from pig and rat livers are at the present time too small for a complete chemical characterization. However, R_f values in four different paper chromatographic systems, infrared and ultraviolet spectra, and reduction in neutral and acidic solutions have been used to obtain preliminary information which enables us to compare these metabolites with the structures of known compounds related to α-tocopherol. The metabolites of vitamin E were located on the paper chromatograms by scanning for radioactivity. In Table I are presented the R_f values of the radioactive metabolites, α-tocopherylquinone, trimethylphytylbenzoquinone, α-tocopherol, and the neutral form of the urinary vitamin E metabolite as determined by the three reverse-phase paper chromatographic systems of Brown,[36] Eggitt and Ward[33] and Lester and Ramasarma,[37] and by the absorption paper chromatographic system of Green et al.[38] Compound Oi and compound F are not Em-

TABLE I

R_f-Values of Labeled Compounds Found in Liver Tissues as Metabolites of C^{14}-Labeled α-Tocopherol and of Some Other Related Compounds

Compound	Brown[36]		Eggitt and Ward[33]			Green[38]	Lester and Ramasarma[37]	
	90/10	50/50	75% EtOH	80% PrOH	95% PrOH		80% PrOH	95% PrOH
α-Tocopherol	0.62	0.00	0.25	0.85	...	0.75	0.83	...
Compound O (insoluble in EtOH)	0.00	0.00	0.00	0.00	0.00	0.85	0.00	0.00
Compound O (soluble in EtOH)	0.00	0.00	0.00	...	0.10	0.85	0.00	0.21
Compound F	0.89	0.30–0.45	0.65	0.88	...	0.36	0.95	...
α-Tocopherylquinone	0.85	0.00	0.50	0.93	...	0.60	0.89	...
α-Tocopherylsuccinate	0.89	...	0.77	...	...	0.00	0.95	...
Trimethylphytylbenzoquinone	0.22	0.00	0.02	0.40	...	0.83	0.46	...
Urinary vitamin E metabolite (neutral form)	S.F.	S.F.	0.88	...	...	...	...	...

TABLE II
Distribution of Radioactivity Expressed as Per Cent

Species	Compound F	Compound O	α-Tocopherol
Pig liver	50.4	1.2	48.4
Rat liver	21.0	5.5	73.5

merie-Engel-positive and differ from each other in their elution patterns on silicic acid-Celite columns, in their ultraviolet adsorption spectra, and in their R_f values in all paper chromatographic systems. Compound Oi and Os (also Emmerie-Engel negative) differ in their ultraviolet absorption spectra, in their solubility properties at low temperatures, and in their R_f values in the reverse phase chromatographic system of Lester and Ramasarma developed with a mixture of 95 per cent n-propanol and 5 per cent water. The R_f values for compounds O and F differ from those of the urinary metabolite of vitamin E, trimethylphytylbenzoquinone, α-tocopheryl succinate and α-tocopherylquinone. Compound F represents the major metabolite isolated from both rat and pig liver, although its absolute amount is extremely small. It is possible to obtain 350 to 400 μg. of crude compound F from a pig liver and 8 to 12 μg. from a rat liver. The conversion of α-tocopherol to compound F and to compounds O was calculated from the percentage of radioactivity recovered for each compound after separation on the columns and indicates considerable conversion of α-tocopherol into compound F (Table II). The O compounds are clearly minor metabolites in both species.

Having found that compound F is a major α-tocopherol metabolite in pig and rat liver, it was important to prove whether or not it is identical with α-tocopherylquinone or trimethylphytylbenzoquinone, which have been considered as major catabolic products[1] or have been suggested as possible functional forms of α-tocopherol.[31] Mixtures of these three compounds were chromatographed on Brown paper and developed with a mixture of 90 per cent acetonitrile and 10 per cent water, and a mixture of 50 per cent acetonitrile and 50 per cent water. In the former case a clear separation was achieved between trimethylphytylbenzoquinone (R_f 0.22) and

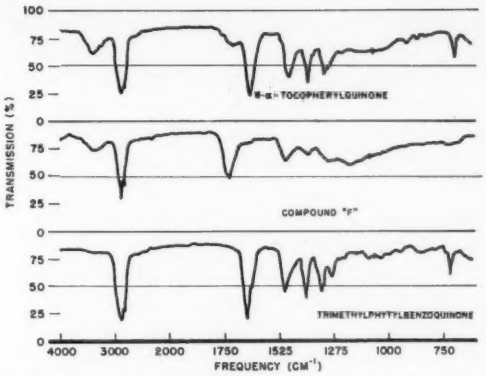

FIG. 8. Infrared spectra of d-α-tocopherylquinone, compound F and trimethylphytylbenzoquinone.

α-tocopherylquinone (R_f 0.85) and compound F (R_f 0.89), in the latter case compound F was separated (R_f 0.3 −0.45) from a mixture of the two quinones (R_f 0.0 and 0.0). In the Eggitt and Ward system, developed with 75 per cent ethanol, a mixture of compound F and α-tocopherolquinone always gave two close but distinct spots (R_f 0.50 and 0.65).

Additional support for a structural difference between compound F and these quinones may be drawn from the infrared spectra (Fig. 8). The typical α,β-unsaturated carbonyl band at 1644 cm.$^{-1}$ present in both α-tocopherylquinone and trimethylphytylbenzoquinone is lacking in compound F. The infrared spectrum of compound F indicated the presence of a hydroxyl group (3400 cm.$^{-1}$), and a very strong band at 1740 cm.$^{-1}$ due to the carbonyl group. There is a slight indication of aromaticity (band at 1600 cm.$^{-1}$, C−C vibration) and strong evidence for the presence of methylene groups (C–H vibrations at 2910 cm.$^{-1}$ and 725 cm.$^{-1}$).

The infrared spectrum of compound Oi is compared in Figure 9 with the infrared spectra of α-tocopherol and α-tocopheryl acetate. On the basis of this spectra it appears that compound Oi contains no hydroxyl group but does contain a carbonyl group.

Compound F and compound Oi were treated with stannous chloride in strongly acidic solution, and with lithium aluminum hydride in neutral solution. The reaction products were isolated and characterized by their R_f values on the Eggitt and Ward system. While compound Oi was unchanged, compound F yielded

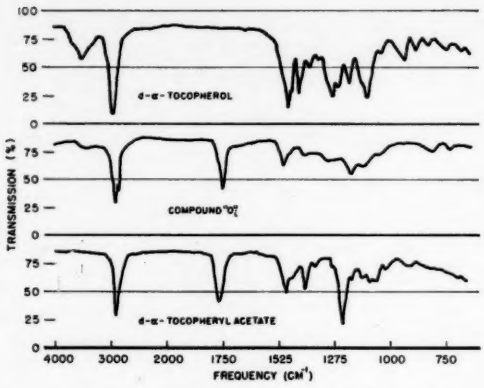

FIG. 9. Infrared spectra of d-α-tocopherol, compound Oi and d-α-tocopheryl acetate.

in both reduction procedures Emmerie-Engel-positive compounds, neither of which was identical with α-tocopherol. In Table III are presented the R_f values of the reduction products of compound F, α-tocopherylquinone and trimethylphytylbenzoquinone. The two latter quinones form α-tocopherol in acidic solution and their respective hydroquinones in neutral solutions.

Neither compound F nor the O compounds show the presence of phosphorus in their molecules as determined by the method of Hanes and Isherwood.[39]

TABLE III

R_f-Values of the Reduction Products of Compound F and Quinones in Eggitt and Ward Paper[33]

Compounds	Reduction with $SnCl_2$ + HCl		Reduction with $LiAlH_4$ (75% EtOH)
	75% EtOH	80% n-propanol	
Compound F	0.00*	0.89*	0.89*
α-Tocopheryl-quinone	0.25†	0.85†	0.00‡
Trimethylphytyl-benzoquinone	0.25†	0.85†	0.00*

* Emmerie-Engel-positive reaction.
† α-Tocopherol.
‡ α-Tocopherylhydroquinone.

COMMENTS

In the isolation procedure which has been briefly described, all necessary precautions were taken to eliminate the possibility of the formation of artifacts. Emmerie and Engel[40] and Moss et al.[41] have shown that tocopherols are sensitive to alkali, but the losses can be diminished in the presence of antioxidants. Mervyn and Morton[26] have studied the effect of pyrogallol as an antioxidant on the analysis of kidney tissue with and without added tocopherol. When the tissue was digested with premixed 60 per cent ethanolic KOH and pyrogallol they were able to recovery 88 per cent of added α-tocopherol. In our experiments on the saponification of labeled α-tocopheryl succinate with the same premixed solution and subsequent purification of the reaction mixture on the silicic acid-Celite column only 40 to 50 per cent of the α-tocopherol was recovered. In contrast, about 95 per cent recovery of α-tocopherol was achieved by reduction of the succinate ester with lithium aluminum hydride followed by silicic acid-Celite column chromatography. In saponification experiments several artifacts were eluted from the silicic acid-Celite column. One of these was identified by R_f values, ultraviolet absorption spectra and reducibility in acidic solution to α-tocopherol as α-tocopherylquinone. Another compound showed an R_f of 0.0 in the Eggitt and Ward system and 0.8 in the system of Lester and Ramasarma (95 per cent n-propanol as developing solvent), and thus could be distinguished from compound O.

In the experiments in which α-tocopheryl succinate was reduced by lithium aluminum hydride and the products were separated by chromatography on a silicic acid-Celite column, only small amounts of these artifacts were obtained. These artifacts were distinguished from the true metabolites by ultraviolet absorption and R_f values. In all our experiments the saponification of tissue or extracts was avoided not only because of the possible degradation of α-tocopherol but also because of the sensitivity of the metabolites to alkali. It was necessary to remove phospholipids by precipitation and to eliminate, stepwise, neutral fats, free and esterified cholesterol, and other fat-soluble substances by using different columns and paper chromatographic systems, since these would contaminate the fractions containing the α-tocopherol metabolite. In contrast to the recent report

by Diplock et al.,[25] but confirming previous findings[11,27–29] we were unable to find any detectable α-tocopherylquinone in rat or pig livers. It is possible that α-tocopherylquinone might be formed in the intestine[24] or in tissues under pathologic conditions,[26] although Mervyn and Morton did not find it in all nephritic kidneys. Uncontrolled tissue saponification leads to the partial destruction of α-tocopherol and to the formation of α-tocopherylquinone and other unidentified artifacts. Our studies have shown that whenever one has to rely upon paper chromatographic methods for identification of extremely small amounts of structurally very similar compounds, several systems must be used. It has been found that by the oxidation of ubichromenol or ubichromanol compounds analogous to α-tocopherylquinone are formed, which show in some reverse-phase chromatographic systems the same R_f values as α-tocopherylquinone.[42]

It has been shown that neither Simon's metabolites nor trimethylphytylbenzoquinone are present in liver tissue and thus cannot be considered as functional forms of vitamin E. The major metabolite, compound F, is neither a quinone nor a hydroquinone, and contains a long saturated carbon chain, a carbonyl group, and possibly a benzene ring in its molecular structure. It can be readily reduced to two different Emmerie-Engel-positive compounds, depending on the reduction method, neither compound being α-tocopherol.

Preliminary studies on the distribution of the α-tocopherol metabolites in subcellular fractions[29] have indicated the presence of compound F and α-tocopherol in liver mitochondria. Although it has been shown that α-tocopherol does not form a redox pair with compound F and does not participate as a carrier in an electron transport system, one may speculate that possibly compound F and its reduction product function as a redox pair either in an electron transport system or in some other biological system. In such a case one might explain the role of α-tocopherol in acting as a physiologic antioxidant and as the starting material for the formation of compound F, which then participates with its reduction product in a specific reaction. Absence of phosphorus in compounds F and O does not support the suggestions for a possible participation of α-tocopherol or its metabolites in oxidative phosphorylation.[11] On the basis of our results it is somewhat difficult to visualize the role of α-tocopherol as only that of an unspecific antioxidant because of the absence of such typical oxidation products as α-tocopheryl-quinone or tocored or tocopurple[43] in animal tissues. These should have been formed had α-tocopherol only been protecting the cellular units from the accumulation of lipid peroxides. Inglett and Mattill[44] and Harrison et al.[17] have demonstrated that the oxidation of α-tocopherol by benzoyl peroxide, cytochrome C or hydrogen peroxide results primarily in the formation of α-tocopherylquinone.

It is hoped that further characterizations of the metabolites (compound F and O) following their isolation in sufficient quantities will throw more light on the *in vivo* function of vitamin E.

THE ISOLATION OF A NEW REDUCING COMPOUND FROM ANIMAL TISSUES

It has been shown that the synthetic antioxidants *N,N'*-diphenyl-p-phenylene-diamine (DPPD)[46] and methylene blue (MB)[45] can prevent and cure the reproductive failure of vitamin E deficiency in female rats.

In further extension of these studies it has been found that rats carried through resorption-gestation on a vitamin E-deficient diet will respond in subsequent reproductive cycles to the antioxidants DPPD and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (Ethoxyquin)[47] bearing live young. The antioxidants prevented the resorption-gestation symptoms of vitamin E deficiency when fed from the beginning of the experiment to rats in E-free rations, and this protective effect was carried through at least two generations when DPPD was continuously included in the diet at levels similar to those required for vitamin E.[46,47,48] Since rats could be maintained and carried through two generations on a vitamin E free diet supplemented with synthetic antioxidants, it became important to determine whether the antioxidant was itself responsible for the cure, or whether it was merely functioning to preserve minimal amounts of vitamin E or its metabolites which might be left in the body tissues.

TABLE IV
R_f Values of Emmerie-Engel-Reactive Compound Present in Liver Extracts

Diet	Treatment	Eggitt and Ward[33]		Silicone[37]
		EtOH (75%)	n-PrOH (80%)	n-PrOH (80%)
Vitamin E free	None	0.00*	0.53*	0.77
Vitamin E free	α-Tocopherol	0.00*	0.53*	0.78
		0.25	0.85	0.84
Vitamin E free	DPPD	0.00*	0.53*	0.77
		0.83	0.87	0.84
Vitamin E free	BHT	0.00*	0.53*	0.77
		0.95	0.95	...
Vitamin E free	Ethoxyquin	0.00*	0.53*	...
		0.84	0.90	...
Checkers	None	0.00*	0.53*	0.74

* These are initial values. Final values after repeated chromatography and purification are 0.79 and 0.90.

Female rats maintained on the vitamin E free synthetic diet[42] were killed at the end of each experiment, and the livers were removed and analyzed for vitamin E.[47,48] Homogenized livers were extracted with foaming solvent in the presence of pyrogallol. The extract was not saponified, because of the destructive action of alkali on α-tocopherol and its metabolites and also because of possible formation of ubichromenol or some other reducing artifacts which could be formed from ubiquinone in boiling alkaline ethanol solution.[49–51] The residue was dissolved in absolute ethanol, and the neutral fats were precipitated by cooling the solution in dry ice-acetone. The filtrate was purified by subsequent reverse phase paper chromatography, using the systems of Eggitt and Ward[33] and Lester and Ramasarma.[37] The R_f values of the Emmerie-Engel reactive compounds found in liver are presented in Table IV. It was found that animals on vitamin E free diets or even on a low E diet (Checkers) contained no detectable α-tocopherol in their livers and carcasses. If α-tocopherol were added to the diet it could be found and quantitatively determined in the animal tissues. Synthetic antioxidants, DPPD, Ethoxyquin (EMQ), and BHT (2,6-di-tert-butyl-4-methyl-phenol) occurred in the liver tissues and could be quantitatively determined when given to the animals. It was found that, in addition to these administered antioxidants, all rats contained an additional Emmerie-Engel-positive material (compound X), whether or not vitamin E or any antioxidant was fed. Furthermore, it was found that the level of compound X in rat liver tissue appeared to be related to the level of α-tocopherol fed.[48] The concentration of compound X in vitamin E deficient rat liver was 9.0 μg./gm. of fresh tissue calculated as vitamin E.

Hines and Mattill,[27] using the Emmerie-Engel assay method for vitamin E in rat and rabbit liver and muscle tissue, showed that the level of vitamin E so determined was related to the level of vitamin E in the diet. The concentrations of total "vitamin E" (total Emmerie-Engel-reactive material) in liver tissues of animals fed high vitamin E, normal, and vitamin E deficient diets were: for the rabbit, 86.8, 9.2 and 9.4; and for the rat, 42.3, 22.1 and 22.6 μg./gm., respectively. These authors assumed that their figures represented only tocopherols. Hickman, Kaley and Harris[52] observed the presence of a substance with reducing properties in extracts of rat and human feces which gave "apparent" tocopherol values when assayed by the Emmerie-Engel method. They found that when the subjects were given higher doses of α-tocopherol the excretion of α-tocopherol and of the other reducing substance increased proportionally to the amount of α-tocopherol administered. Without any further studies on the identification of this substance, they concluded that it was a labile substance spared by the tocopherol in the alimentary tract. Rosenkrantz, Milhorat and Farber[24] confirmed the presence of a heat-labile, alcohol-soluble reducing substance in human stools but attempts to identify this material were unsuccessful. Bolliger and Bolliger-Quaife[53] reported that chromatography of various tissue extracts gave two Emmerie-Engel-reactive compounds of which only one was α-tocopherol. Very recently, Bieri and his co-workers[54,55] have found that liver and heart tissues of chickens raised on vitamin E free diets contained Emmerie-Engel-reactive material which did not chromatograph as α-tocopherol.

Slater and his co-workers[11] have shown that the increase in apparent α-tocopherol content of tissues analyzed by the method of Bouman

TABLE V
R_f Values of Emmerie-Engel-Reactive Compounds

Compounds	Eggitt and Ward[33]		Silicone[37]	Green[38]
	EtOH (75%)	n-PrOH (80%)	n-PrOH (80%)	
α-Tocopherol	0.25	0.85	0.85	0.75
DPPD*	0.83	0.95	0.84	0.82
Ethoxyquin†	0.84	0.90	0.91	...
BHT	0.95	0.95	0.80	...
Ubichromenol-50	0.00	0.40	...	...
Ubichromanol-50	0.00	0.34	0.62	0.73
Ubiquinol-50	0.00	...	...	0.73
Compound X	0.00 (initial)	0.53 (initial)	0.80–0.90	0.78
	0.79 (final)	0.90 (final)	...	...

* N,N′-diphenyl-p-phenylenediamine.
† 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (Santoquin).

et al.,[56] which employs a preliminary treatment of the tissue extract with ascorbic acid in HCl, was due to the formation of acid-reduced ubiquinone, an Emmerie-Engel-positive cyclization product of ubiquinone. The same compound was found to be present in all extracts which were passed through the Florex XXS column.[48,57] Laidman et al.[58] have isolated and identified an Emmerie-Engel-reactive compound, present in the unsaponifiable matter of kidney and other tissues of many species as a cyclic isomeride of ubiquinone 50 (ubichromenol) which contains a chromene ring and a side chain consisting of nine isoprenoid units. Diplock et al.[59] have found that the mixture of ubiquinones 50, 45, 40 and 35 in rat tissues is accompanied by a similar mixture of ubichromenols. They observed that ubichromenol is present only if the preparation contains the corresponding ubiquinone. Ubihydroquinones (ubiquinols),[60] ubichromenols, ubichromanols, vitamin A and carotenoids are so far the only well characterized nontocopherol reducing compounds possibly present in animal tissues. The natural occurrence of ubichromenols is still unsolved,[49–51] and ubichromanols if present in extracts must be considered as artifacts. The R_f values of these reducing compounds are given in Table V.

The new unknown reducing substance,

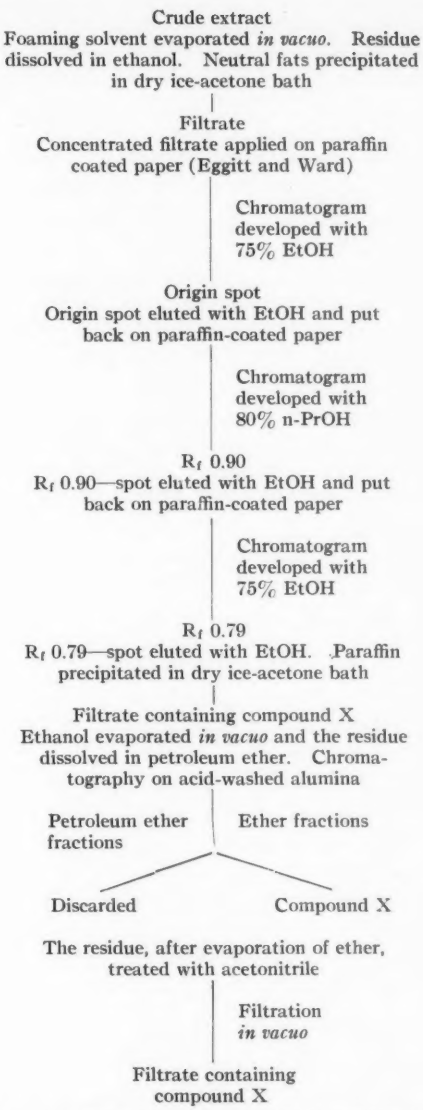

FIG. 10. Flowsheet for the isolation of compound X.

compound X, differs distinctly from ubichromenol, ubichromanol and tocopherols in reverse phase paper chromatographic systems. This compound has been found in rat, pork, beef and chicken liver and beef heart mitochondria. The isolation procedure is outlined in Figure 10. It does not include alkaline saponification, excessive heat or column chromatography on Florex XXS, avoiding thus the conditions for the possible formation of ubichromanol or other artifacts. The neutral

fats, cholesterol and cholesterol esters are separated by precipitation from ethanol solution in dry ice-acetone, and vitamin A, carotenoids and ubichromenol are then separated from compound X on paper. The final purification is achieved by chromatography on a deactivated (10 per cent) alumina column. Compound X is a pale yellow oily substance characterized by: an inflexion at 271 mμ. and R_f values of 0.79 and 0.90 in Eggitt and Ward system chromatograms (developed with 75 per cent EtOH and 80 per cent n-propanol, respectively); 0.80 on silicone-treated paper (developed with 80 per cent n-propanol); and 0.78 in the Green system. Its infrared spectrum shows the presence of a hydroxyl group (3400 $cm.^{-1}$) and a strong C=O band (1735 $cm.^{-1}$). Green et al.[51] suggested that compound X may be ubichromenol 45 or 50. The presence of a strong carbonyl band, different R_f values and a different ultraviolet absorption differentiate compound X from the ubichromenols. Very recently, Brand, Dahl and Mahler[61] reported the presence of a rapidly migrating component with an R_f value 0.90 − 0.93 on the paper system of Lester and Ramasarma, developed with 80 per cent n-propanol. This material, which was not reduced by KBH_4 but was capable of interacting with neotetrazolium chloride, may be similar to or identical with compound X. The authors considered their compound to be ubichromenol or a derivative of it, such as phosphate ester. It has been found that compound X does not contain phosphorus.

There is no precursor product relationship between compound X and α-tocopherol, since compound X is not labeled following administration of C^{14}-α-tocopherol. It may be possible that compound X is an important functional compound in the body and that vitamin E and other active antioxidants function in the reduction of this compound to this active form or in the protection of this compound, which is formed by the animal body.

SUMMARY

It has been demonstrated that α-tocopherol-5-methyl-C^{14} was converted in rats and pigs into three metabolites: compound F and compounds Oi and Os. These compounds are oils, and they were isolated and separated from liver extracts by a combination of column and paper chromatographic methods. Compound F, which is the main metabolite, can be reduced readily to an Emmerie-Engel-positive material. Neither compound is a quinone or hydroquinone, and neither contains phosphorus in its molecule. It has been shown that the compounds α-tocopherylquinone, α-tocopherylhydroquinone, α-tocopheroxide, trimethylphytylbenzoquinone and Simon's metabolites, which have been suggested as possible metabolic or degradation products of α-tocopherol, are not present in rat and pig liver under conditions used in these experiments. The importance of these compounds in studying the function of vitamin E has been discussed.

The isolation of a new reducing compound from rat, chicken, and beef liver and from beef heart mitochondria has been described and its possible relationship with α-tocopherol and other antioxidants discussed.

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The Nature of the Action of Selenium in Replacing Vitamin E

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RECENT advances in our knowledge of vitamin E metabolism in the chick have contributed greatly to a better understanding of the function of this vitamin, and of the interrelationship between the vitamin and other nutrients. For example, it has been shown that under some dietary conditions the young chick grows and develops normally without dietary vitamin E.[1] It is now well established, however, that in the absence of vitamin E the production of the various vitamin E deficiency symptoms in this species can be controlled at will, primarily by alterations in the type and amount of dietary polyunsaturated fatty acids[2] but also by the inclusion or omission of dietary selenium[3,4] or cystine.[5]

It has also been established that all vitamin E deficiency syndromes in the chick (encephalomalacia, exudative diathesis and white muscle striations) can be prevented by antioxidants which are chemically unrelated to α-tocopherol.[6,7] It thus appears that in this species the sole biochemical function of vitamin E may be that of a cellular antioxidant. With this background, we decided to investigate the possibility that the mode of action of selenium and cystine, which can replace vitamin E under some circumstances, may be due to an unrecognized antioxidant effect of these substances in the body.

To test this possibility we have used thiobarbituric acid for determining the occurrence of lipid peroxidation. In the autoxidation of polyunsaturated fatty acids one end product is malonic dialdehyde. This substance reacts with thiobarbituric acid to give a red compound with an absorption maximum at 535 mμ.[8]

EXPERIMENTAL

In our experiments, chicks one to five days old were fed purified-type vitamin E free diets with various supplements for 28 to 35 days (Table 1). They were then sacrificed and the tissues removed and stored at $-20°$C. Five per cent homogenates were prepared in phosphate buffer of pH 7.4. Two to five milliliters were incubated in air, with shaking, at 37°C. At intervals, aliquots of the homogenates were removed and the reaction with thiobarbituric acid performed.[9]

The peroxidation of the tissue lipid in homogenates is essentially a nonenzymatic reaction and proceeds to a similar extent in heat-inactivated preparations.[9] Under the conditions of our experiments, with no additions of cofactors or substrates, there is no detectable respiratory activity.

RESULTS

Our first experiments, using Diets A and B (Table 1), were limited to the liver and muscle. The significant components of these diets with regard to the deficiency symptoms produced should be emphasized. Diet A contained 30 per cent of soybean protein, 1 per cent of stripped lard and 0.3 per cent of L-cystine. This diet has weak pro-oxygenic activity and produces a low incidence of exudates. No encephalomalacia or white muscle striations appear under these conditions. Diet B contained 15 per cent of casein, 10 per cent gelatin and 4 per cent of stripped lard but no added cystine. It is moderately pro-oxygenic and produces a high incidence of white muscle striations. The casein contained sufficient bio-

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TABLE I
Composition of Vitamin E Free Diets for Chicks

Ingredient	Diet A	Diet B	Diet C
Soybean protein*	30	...	...
Purified casein	...	15	...
Torula yeast†	...	...	60
Gelatin	...	10	...
"Stripped" lard‡	1	4	4
Salt mixture	6	6	6
L-Cystine	0.3	...	...
DL-Methionine	...	...	0.3
Glycine	...	...	1.0
Vitamin mixture§	0.2	0.2	0.2
Glucose	62.5	64.8	28.5

* Assay protein C-1, Archer-Daniels-Midland Company.
† Feed grade, Lake States Yeast Corp.
‡ Distillation Products Industries.
§ Provides adequate amounts of all vitamins except vitamin E.[14]

logically active selenium to prevent exudates. No encephalomalacia occurs with this level of lard (insufficient linoleic acid).

In Table II are given the thiobarbituric acid values (TBA values) from one trial in the first series of experiments.[10] Two other trials gave similar results. When the tissues from chicks receiving dietary vitamin E were incubated essentially no peroxides were formed. (Low TBA values of 35 or less do not represent the pink compound formed from TBA and malonic dialdehyde, but are due to a faint yellow color given by most tissues.) In contrast, the tissues from vitamin E depleted chicks formed considerable quantities of malonic dialdehyde, with liver having higher quantities than muscle. In the group given selenium the TBA value was less than one-half that of the vitamin E deficient group; i.e., peroxidation was significantly reduced by feeding selenium. There was no effect, however, on the muscles in this group.

In the chicks fed Diet B without supplementation, a high incidence of white muscle striations occurred. The muscles in this group gave TBA values significantly higher than those from chicks receiving 0.3 per cent L-cystine. There was no effect of the cystine on the TBA values for liver. In the muscles deficient in both vitamin E and cystine (Group 4) there was no correlation between the severity of striations and the TBA values. In the

TABLE II
Effect of Dietary Selenium and Cystine on Thiobarbituric Acid Values of Incubated Chick Tissues

Group No.	Diet	No. of Chicks	Addition to Diet	Average TBA Values*	
				Liver	Muscle
1	A	7	None	185	90
2	A	7	0.5 p.p.m. Selenium	65†	75
3	A	4	0.01% α-Tocopheryl acetate	40†	35†
4	B	7	None	150	85
5	B	7	0.3% L-Cystine	125	60†
6	B	3	0.01% α-Tocopheryl acetate	35†	20†

NOTE: 5 per cent homogenates incubated for one hour at 37°C.
* $A_{535} \times 1{,}000$.
† Significantly lower than control group ($P < 0.01$).

group fed tocopherol, TBA values were very low as with Diet A.

In a subsequent series of experiments, which were conducted in another laboratory,* the aforementioned observations with Diets A and B were extended to tissues other than liver and muscle. In addition, a third diet (C, Table I), which contained 60 per cent of Torula yeast as its most significant component with regard to vitamin E deficiency, was also tested. Diet C is a strongly pro-oxygenic diet, due primarily to the high linoleic acid content of the yeast,[11] and produces a high incidence of severe exudates as well as occasional encephalomalacia.

The TBA values obtained from incubated tissue homogenates from chicks fed the soybean protein diet (A) are shown in Table II. As found previously in our laboratory,[9,10] and also by others,[7,12,13] when the diet contained sufficient α-tocopherol or other antioxidant (in this case ethoxyquin†), little or no peroxidation of the tissue lipids occurred. When 0.33 p.p.m. selenium was in the diet a significant reduction ($P < 0.01$) in TBA values was observed in the liver, kidney and heart. It is thus apparent that the antioxidant action of dietary selenium is not restricted to the liver.

* In the laboratory of Professor H. Dam, Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark.
† 1,2 - dihydro - 6 - ethoxy - 2,2,4 - trimethylquinoline, Santoquin,® Monsanto Chemical Co., St. Louis, Missouri.

TABLE III

Thiobarbituric Acid Values in Homogenates of Tissues from Chicks Fed Diets Containing 30 Per Cent Soybean Protein (Diet A)

Group No.	Addition to Diet	Average TBA Values for Various Tissues*					
		Liver	Kidney	Muscle	Heart	Lung	Spleen
1	None	105	130	45	80	40	150
2	0.01% *dl*-α-Tocopheryl acetate	20†	35†	30	15†	15†	10†
3	0.1% Ethoxyquin	30†	10†	35	20†	30†	90†
4	0.33 p.p.m. Selenium	40†	70†	35	55†	40	110
5	0.3% L-Cystine	95	120	30	85	55	150

NOTE: Five per cent homogenates incubated one hour at 37°C. Nine to sixteen chicks per group.
* $A_{535} \times 1{,}000$.
† Significantly lower than Group 1 ($P < 0.01$).

TABLE IV

Thiobarbituric Acid Values in Homogenates of Tissues from Chicks Fed Diets Containing 60 Per Cent Torula Yeast (Diet C)

Group No.	Addition to Diet	Average TBA Values for Various Tissues*					
		Liver	Kidney	Spleen	Muscle	Lung	Brain
1	None	360	130	230	35	25	410
2	0.01% *dl*-α-Tocopheryl acetate	20†	65†	20†	0	0	440
3	0.1% Ethoxyquin	20†	0	30†	0	0	...
4	1 p.p.m. Selenium	130†	120	230	35	120	460
5	0.1 p.p.m. Selenium	100†	130	200	45	140	500

NOTE: Five per cent homogenates incubated for one hour at 37°C. Seven chicks per group.
* $A_{535} \times 1{,}000$.
† Significantly lower than Group 1 ($P < 0.01$).

There was no effect of cystine in this diet on the TBA values.

In a second experiment, using the Torula yeast Diet C, somewhat different results were obtained (Table IV) from those with the soybean protein diet. The greater pro-oxygenic activity of Diet C is reflected in the higher TBA values for liver in the unsupplemented group (360 versus 115 for Diet A). Both α-tocopherol and ethoxyquin again afforded antioxidant protection so that little or no peroxidation occurred. (The case of brain is discussed hereafter.) The somewhat higher TBA values for the vitamin E supplemented group, compared with the same group fed Diet A, is probably a reflection of the poorer utilization of an α-tocopherol when Torula yeast is fed.[14]

When either 1 or 0.1 p.p.m. selenium was fed in this diet a significant decrease in the TBA value from liver was observed. In the kidney, however, the decrease in TBA value produced by selenium in the soybean protein diet was not found with the Torula yeast diet. This may be due to the greater pro-oxygenic activity of the Torula yeast.

It is interesting that a tenfold increase in the dietary level of selenium produced no additional reduction in the amount of peroxidation. This suggests that the observed effect of selenium is not due to the total selenium content of the tissue but probably is due to a functional form which is present in optimum amount even at the lowest dietary level.

An unexplained phenomenon found in this experiment was the increase in TBA value in the lungs from chicks fed selenium. This was not observed with the other two diets. It is possible that the Torula yeast diet caused an increase in deposition of inorganic selenium in the lungs; we have found that inorganic selen-

TABLE V
Effect of Selenium and Cystine Added to Liver Homogenate on Subsequent Lipid Peroxidation

Addition to Flask*	TBA Value
None	99
5 μg. Selenium	100
12.5 μg. Selenium	119
25 μg. Selenium	265
50 μg. L-Cystine	100
125 μg. L-Cystine	90
250 μg. L-Cystine	93

* 2 milliliters of a 5 per cent homogenate of liver from a vitamin E deficient chick (Diet B) in phosphate buffer pH 7.4, incubated for one hour at 37°c. and TBA value determined as described in the text. Values are averages of duplicate flasks. Selenium added as sodium selenite.

ium added to homogenates of vitamin E deficient liver exerts a pro-oxidant effect (Table V) at a relatively high concentration. It can be seen that added cystine has no significant effect on the TBA value.

The high TBA values produced by brain have been commented on previously.[10] Similar values were noted in these experiments for all brains, regardless of the diet fed or whether or not α-tocopherol, antioxidants or other supplement were fed. The extensive lipid peroxidation which occurs in incubated brain homogenates is probably a result of the high polyunsaturated fatty acid content of this organ[15] coupled with its relatively low content of α-tocopherol.

In the third experiment of this series, the TBA values were determined in incubated tissue homogenates from chicks ingesting Diet B which contained 15 per cent purified casein, 10 per cent gelatin and 4 per cent lard. As seen in Table VI, the degree of peroxidation was generally lower than that obtained with either of the two previous diets. It should be restated that the casein in this diet contained biologically active selenium; this may explain the extremely low TBA value found in liver.

Tocopherol, as with the other diets, reduced the TBA values in all tissues. The feeding of selenium, however, had no effect on the extent of peroxidation in any of the tissues investigated; presumably this was because maximally effective levels of the element were already present in the basal diet.

In the group supplemented with L-cystine, a significant decrease in TBA value was obtained only for muscle, in confirmation of the earlier experiments noted above.

COMMENTS

It is apparent from these studies that dietary selenium, and to a lesser extent cystine, in some manner alter the composition of tissues with the result that autoxidation of the lipids *in vitro* is significantly reduced. Inasmuch as the only definitely established function of α-tocopherol in metabolism is its antioxidant action, it is appealing to suggest that selenium and cystine act by a similar mechanism. In view of the *in vitro* nature of these studies, however, it is obvious that more definitive work is necessary before such a conclusion can be drawn.

The autoxidation of tissue lipids under conditions such as described here is indeed a complex process. In its simplest form, it may be stated that the polyunsaturated fatty acids in the

TABLE VI
Thiobarbituric Acid Values in Homogenates of Tissues from Chicks Fed Diets Containing 15 Per Cent Casein and 10 Per Cent Gelatin

Group No.	Addition to Diet	Average TBA Values for Various Tissues*					
		Liver	Kidney	Muscle	Heart	Spleen	Lung
1	None	40	60	35	65	100	45
2	0.01% *dl*-α-Tocopheryl acetate	20†	25†	5†	10†	30†	10†
3	0.33 p.p.m. Selenium	50	60	25	45	130	40
4	0.3% L-Cystine	50	60	20†	50	65	40

NOTE: Five per cent homogenates incubated for one hour at 37°c. Eight to ten chicks per group.
* $A_{535} \times 1{,}000$.
† Significantly lower than Group 1 ($P < 0.01$)

presence of oxygen and suitable catalysts and activators undergo peroxidation not appreciably different from the classical concepts obtained with model systems. It has been shown in the case of tissue homogenates or cell fractions, however, that a number of complicating factors may alter the course of the lipid autoxidation. Thus, the addition of certain substrates[16] or of certain metallic ions[13] or complexing agents[17] decrease *in vitro* peroxidation, while other substances[18] increase it. Although similar effects may or may not occur *in vivo*, it remains to be shown just what metabolic conditions, other than a deficiency of vitamin E, significantly alter the tendency toward peroxidation in the intact tissue.

Recently, the concept of an "antioxygenic potential" in tissues, the balance of prooxidant versus antioxidant factors, was reintroduced into the problem of vitamin E function.[9] Although this is not a new concept, having been described earlier, for example by Hickman[19] as the "antioxidant balance," it is undoubtedly a real phenomenon which unfortunately has not been adequately recognized in experimental vitamin E work. In terms of this concept, dietary selenium and cystine increase the antioxygenic potential of the tissues.

It was pointed out previously that the observed antioxidant effects of selenium and cystine are not due simply to increased concentrations of these substances as such, in the cells. It was shown in the case of selenium that a tenfold difference in dietary level did not alter the degree of antioxidant protection in the liver. This would suggest that either the incorporation of biologically active selenium into liver is limited, or that minimal amounts of the active form are sufficient for the antioxidant effect. In view of the intimate association of selenium with the sulfur amino acids[20] it may be hypothesized that an alteration of normal proteins by their content of selenium, in some way increases the antioxygenic potential of the cell.

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DISCUSSION

Dr. Lawrence J. Machlin, (*St. Louis, Missouri*): Before discussing the role of selenium I would like to review our concept of the etiology of encephalomalacia since it relates to the theory of lipid peroxidation discussed by Drs. Caputto and Bieri. We have found[1] that chickens fed purified diets containing no antioxidant and a high level of linoleic acid will develop encephalomalacia. Under these conditions chickens grow as rapidly as control animals receiving antioxidants. The first apparent sign of pathology is a slight ataxia. Thereafter the progress of the disease is extremely rapid, usually resulting in death in less than twenty-four hours with typical drastic disruption of cerebellum cellular integrity. Selenium and/or sulfur amino acids have no effect, whereas a number of antioxidants of unrelated chemical structures will completely prevent the development of this symptom. The proportion of linoleic and arachidonic acids in cerebellum lipids increases when linoleic acid is added to the diet.[2] The first incidence of the disease is usually at twelve days of age. This coincides with the time it takes for arachidonic and linoleic acids content to increase in the cerebellum from the levels observed at hatching to a maximal value. During this time it is also known that tissue levels of vitamin E are rapidly being depleted (0 to twelve days). By twelve days the vitamin E level in the cerebellum is evidently no longer high enough to protect the increased level of linoleic and arachidonic acid and therefore these acids (and perhaps other highly unsaturated acids of the linoleic acid family) then autoxidize in a manner similar to mechanisms proposed for their autoxidation *in vitro*. This could result in either the breakdown or weakening of certain structural units such as the cell walls of the blood vessels or in the interference with reactions catalyzed by lipoprotein containing enzymes such as occur in the mitochondria.

In regard to the role of selenium we agree with Dr. Bieri that this element can function as an antioxidant in animal tissue. In our studies[3,4] high levels of an antioxidant prevented exudative diathesis (a selenium deficiency). In addition lambs fed Torula yeast Piets developed muscular dystrophy and hydropericardium. Both symptoms are completely prevented by Santoquin®* but are only partially alleviated by selenium.

Dr. Bieri reported that tissues of chickens fed selenium have higher antioxidant activity than deficient chickens. This observation has recently been confirmed by Zalkin et al.[5] These workers have also demonstrated that with no incubation, liver or heart tissues from chickens fed diets supplemented with selenium, contained significantly less TBA reactive material than controls from deficient diets. They interpret this as evidence for the *in vivo* production of lipid peroxide in the tissues of vitamin E and selenium deficient animals. They have also found that certain organic selenium compounds have antioxidant activity in model systems. Selenium compounds are known to be more effective antioxidants then corresponding sulfur compounds.[6] It is quite apparent selenite is converted to an organic compound or compounds which imparts antioxidant activity to tissues. Both Dr. Bieri and Dr. Zalkin and his co-workers suggest that selenoamino acids synthesized from selenite and then incorporated into protein are the active organic compounds. However, except for selenocystine and selenomethionine almost nothing is known about the structure of other selenium compounds in animal tissues. McConnell[7] has found that over 50 per cent of Se^{75} activity in the livers of dogs fed Se^{75} was in the nonprotein fraction. He found Se^{75} in alcohol and ether extracts of tissue and in a volatile fraction. It is evident that there are unidentified selenium compounds in animal tissues and they should be investigated as possible active metabolites concerned in vitamin E or antioxidant deficiencies.

Dr. Klaus Schwarz (*Bethesda, Maryland*): Having discovered the protective effect of vitamin E on dietary liver necrosis in 1943[8] and that of Factor 3 in 1950,[9] I can assure you that I have spent much time and effort trying to think out and to work out the mutual interrelationship of the two. The more I have done so, the more I have come to the inevitable conclusion that these are two fully independent dietary agents, each acting in its own right.[10] The only obvious connection between the two is the fact that the simultaneous lack of both leads to a special category of diseases such as dietary necrotic liver degeneration in the rat, multiple necrotic degeneration in the mouse, some forms of white muscle disease in calves and lambs, hepatosis dietetica in pigs and others. All of these diseases are fatal. Logically enough, application either of vitamin E or of Factor 3-selenium, independently, prevents their development. A similar relationship is known to exist between folic acid and vitamin B_{12} with respect to certain forms of anemia. For this type of disease, derived from simultaneous lack of two independent dietary components, I suggested the term "ambogenous" over fifteen years ago in the European literature.[11]

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* 1,2 dihydro-6-ethoxy 2,2,4 trimethylquinoline, an antioxidant, is manufactured by the Monsanto Chemical Company.

Thinking in these simple and what I believe to be reasonable terms, one may take issue with the title of Dr. Bieri's talk in which he uses the term "replacement of tocopherol by selenium." Problems of definition and of semantics are at stake here, of course. It is almost certain that he means something different by "replacement" than I do when using this term. To me replacement would indicate actual substitution at the site of action within the organism in intermediary metabolism, i.e., in the last analysis in catalytic systems. Such a replacement apparently does not take place.

The well established concept of *alternate pathways* in intermediary metabolism would offer a variety of possibilities to interpret the relationship between vitamin E and Factor 3-selenium. It has been our working hypothesis that the two factors may act independently in alternate pathways of energy metabolism, for instance, in independent chains of electron transfer, and that both of these pathways must be impaired before a fatal, ambogenous deficiency disease evolves.[11]

I not only doubt that Factor 3-selenium "replaces" vitamin E, I also believe that peroxidation and antioxidant activity may not be the mechanisms through which these diseases are caused or prevented. There is no doubt, of course, that unsaturated fatty acids and other unsaturated components accelerate all kinds of vitamin E deficiency diseases, as shown originally by Evans and Burr,[11] and by Mattill,[12] and confirmed innumerable times by others including myself.[13] This does not justify the conclusion that prevention of rancidity is the true metabolic function of vitamin E in the living organism.[14] This conviction is based on thorough comparative studies of a series of thirteen different antioxidants, and many other studies on the metabolic aspects of the latent phase of liver necrosis, carried out by our group since 1952. If I may be permitted to outline the main findings with respect to the argument at hand, they are as follows:

(1) The thirteen antioxidants were first tested *in vivo* against liver necrosis, i.e., they were fed to the animals. Some of them were indeed similar to tocopherol in protecting against the disease. However, all those commonly used for the stabilization of fats against peroxidation were ineffective.[15] The active compounds were either quinonoid structures or, like DPPD, tend to exist in solution largely as free radicals. Subsequently, we assayed all thirteen compounds against respiratory decline, a characteristic metabolic impairment of liver tissue during the latent phase of dietary necrotic liver degeneration, demonstrable ten to fourteen days before the development of necrosis. The compounds were applied in the following two ways: by injection into the portal vein immediately before extirpation of the liver,[16] and by direct *in vitro* addition to the liver slices in the Warburg medium.[17] In both systems, the results were generally in line with those obtained in the previous series of experiments. They showed clearly that antioxidant activity as such was not the essential criterion for protection against the metabolic defect.

(2) A comparison of results obtained by these three methods of application (dietary, intraportal and *in vitro*) disclosed major inconsistencies in the effects of two substances, methylene blue and α-tocopherol. Methylene blue was highly effective when present in the oxidized blue state, for instance, when added to the Warburg medium. When reduced to the leuko-form, for example after intraportal injection, it was inactive. Yet, it is leukomethyleneblue that acts as an antioxidant, while the oxidized state is useless for the antioxidant function. Tocopherol, on the one hand, was highly effective in the diet, and also potent when injected intraportally, but it was completely inactive when added directly to the Warburg medium. The Simon tocopherol metabolite, on the other hand, containing a quinonoid structure and a much shortened, profoundly altered side chain, was highly effective when added directly. Here again, the effective compounds were quinone structures which are not directly active as antioxidants, while α-tocopherol has antioxidant capacity but was inactive. These results led us to the postulate that tocopherol is converted into an active form before exerting its metabolic function *in vivo*.[14]

(3) During more recent studies carried out by Dr. L. Corwin in our laboratory,* it has become possible to investigate respiratory decline in homogenates of livers from rats on our Torula diet.[18–20] In the homogenate system, hydroperoxide formation as assayed by the TBA reaction can readily be separated from the specific metabolic impairment. Respiratory decline is prevented by levels of DPPD which are too small to prevent the formation of the TBA-active end products of autoxidation. Peroxidation of the homogenate is in full progress while the characteristic breakdown of respiratory enzyme systems is prevented. About .001 μg. of DPPD per 3 ml. of medium and 50 mg. of homogenized liver suffice to produce this phenomenon. The inverse situation is obtained by direct addition of α-tocopherol to the medium. Even at levels of 1,000 μg. per vessel, α-tocopherol itself is completely without effect on respiratory decline,† while it prevents the development of hydroperoxides and of TBA-inactive breakdown products. This lack of effect of unmetabolized α-tocopherol is in full agreement with the above mentioned results in the slice system. Likewise, in the homogenate as in the slice experiment, the Simon tocopherol metabolite and possibly other tocopherol derivatives prevent respiratory decline, even though they may not have antioxidant properties.

(4) In our laboratory, the thiobarbituric acid reaction has been used by Dr. Corwin to study the question of peroxidation in tissues and tissue homogenates of

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† Previous reports on the effect of α-tocopherol, when added directly to the medium, on respiratory decline in this system were erroneous. Careful examination showed that the earlier studies had been inadequately controlled. The ethanol, used for supplementation of the α-tocopherol, was found effective in this system.

rats on the necrosis-inducing Torula diet and other regimens. Like Dr. Bieri, as well as others, he has not been able to show elevated levels of thiobarbituric acid-active material in vitamin E (as well as Factor 3) deficient animals, even in advanced phases of liver necrosis, provided that the organs were tested immediately after extirpation. It must be emphasized that in Dr. Bieri's experiment, peroxidation takes place *in vitro* in homogenates of organs, and this phenomenon is indeed accelerated in vitamin E deficiency. The same type of result is obtainable in liver homogenates of vitamin E deficient rats during the latent phase of necrotic degeneration. However, to conclude from such *in vitro* observations that the mode of action of tocopherol is merely that of an *in vivo* antioxidant may seem unwarranted if due consideration is given to the facts presently discussed; most of these, incidentally, are contained in previous publications from our group.

From results indicating that the antioxidant hypothesis may not be the final answer to the inquiry into the function of vitamin E within the organism, let me return to the question of replacement of tocopherol by Factor 3-selenium. Dr. Bieri showed, as has Tappel, that not only tocopherol, but also feeding of selenite-selenium slightly delays hydroperoxide formation in tissue homogenates of vitamin E deficient chicks. This does not happen in the rat.[19] As a matter of fact, with rats selenium slightly but significantly *enhanced* the *in vitro* rate of peroxide formation in liver homogenates. It is clear, therefore, that the observations made on chick tissue homogenates cannot be generalized.

For these reasons we may need to search for alternative possibilities in the interpretation of the results obtained with chick tissues. It comes to mind that Dr. Bieri's observations could be explained by differences in the levels of free iron or other trace elements enhancing peroxidation. Tocopherol has an influence on the metabolism of iron. The vitamin is necessary, for example, for the formation of the yellow, iron-containing pigment of the incisors of the rat. Also, as a consequence of their role in energy metabolism, both vitamin E and selenium could profoundly affect the *in vitro* stability of iron-containing structures of the cell; for example, lysosomes. The latter organelles are known to accumulate iron in form of hemosiderin granules. It would be interesting to determine the levels of free and bound iron in tissue homogenates after feeding of diets with and without vitamin E or selenite-selenium.

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The Metabolic Relationships between the Different K Vitamins and the Synthesis of the Ubiquinones

CARL MARTIUS, PH.D.*

IT IS WELL KNOWN that a considerable number of compounds exhibit antihemorrhagic properties in biologic tests. One of these is methylnaphthoquinone (Menadione, vitamin K_3). Related compounds which can easily be transformed into methylnaphthoquinone in the body, such as aminomethylnaphthol ("vitamin K_5") are further examples. In addition to these relatively hydrophilic compounds there is a definitely lipophilic group of active substances such as vitamin K_1 (Phylloquinone) and the series of the K_2 vitamins isolated from bacteria. All of these compounds have practically the same activity on a molecular basis, a rather surprising fact when one considers their considerable difference in solubility and other physical properties. It thus appears improbable that these various substances are active as such, that is without undergoing some previous deep-seated change in the body. *A priori*, two explanations are possible: phylloquinone or the bacteria-vitamins lose their side chain in the organism and are transformed into methylnaphthoquinone, which is then responsible for the physiologic effect. Conversely it may be assumed that the organism has the capacity of introducing a long polyisoprene chain into methylnaphthoquinone transforming menadione into phylloquinone or a vitamin of the K_2 series.

As we have shown experimentally some time ago, the second hypothesis is correct.[1] When suitable experimental animals, preferably chickens raised on a vitamin K free diet are fed labeled menadione, it is possible to extract from the lipophylised organs, alongside the unchanged labeled compound, varying amounts of what has been shown to be vitamin $K_{2(20)}$: 2-methyl-3-geranylgeranyl-1,4-naphthoquinone.[2] To prove its nature we have used a method which has given very good results for the identification of microgram quantities of radioactively labeled compounds of this kind. The extract containing the unknown substance was put through a countercurrent distribution over thirty-five steps using glycolmonomethylether and heptane as the two phases. By measuring the radioactivity of each tube a distribution curve is obtained which usually contains one or more clearly distinguishable peaks. The peaks can be ascribed to individual components by comparison with parallel or mixed distribution experiments carried out with known components. Further confirmation of identity is afforded by running analogous distribution experiments with derivatives of the unknowns and of the standards differing as far as possible in their distribution pattern from the original material. A large number of test runs has shown that the results obtained by this method are very reliable.

We consider the fact that methylnaphthoquinone is transformed in the body into vitamin $K_{2(20)}$ as a proof that the latter compound is the biologically active principle.

There now arises the question of how the living animal cell is capable of carrying out a chemical transformation of a compound which in itself has never been observed in nature and is consequently regarded as a synthetic product. The easiest explanation is to assume

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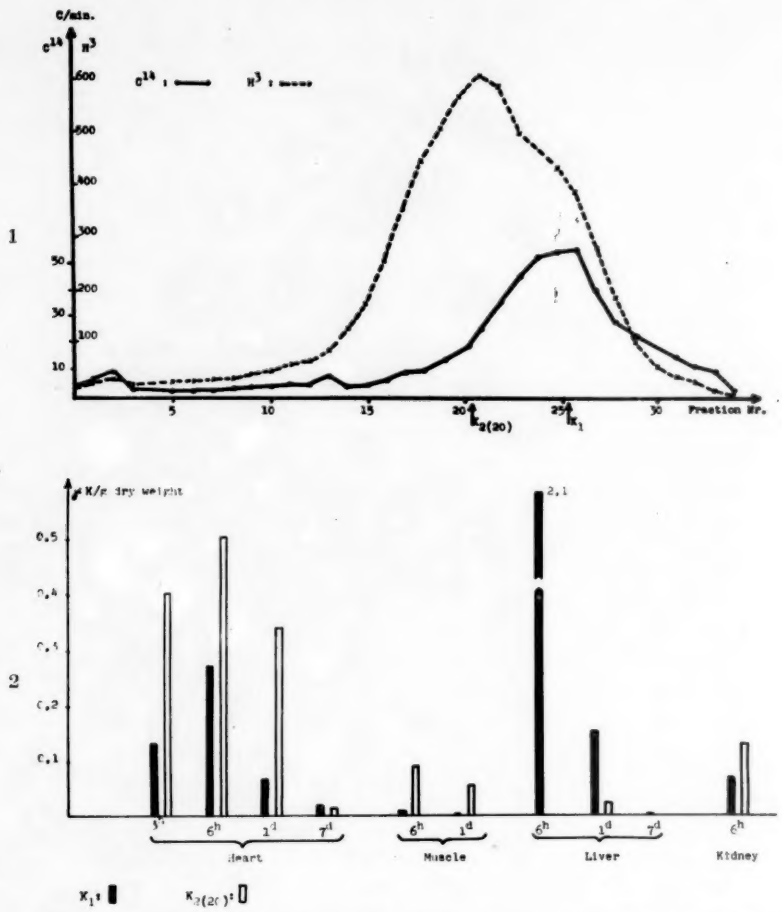

FIG. 1. Countercurrent distribution pattern. Heart extract. Chicken, killed twenty-four hours after application of vitamin K_1.

FIG. 2. Comparison of vitamin K_1 and $K_{2(20)}$ content in heart, muscle, liver and kidney in different time intervals following application of vitamin K_1 (350 μg.). Vitamin K deficient chicken, killed three, six, or twenty-four hours or seven days after application of vitamin K_1.

that methylnaphthoquinone can in fact occur in the cell, for instance as a degradation product of phylloquinone or of other vitamins of the K_2 group. This hypothesis was experimentally tested and proved to be right.[3] Double labeled phylloquinone of high specific radioactivity was synthetized for this experiment. It contained tritium in the methyl group of the nucleus and C^{14} in positions 1′ and 2′ of the phytol side chain. The experimental animals, chickens or pigeons, were fed the labeled compound per os, and were examined after several days. By using the same technic as in the previous experiments it was found that the organs of the animals contained vitamin $K_{2(20)}$ labeled only in the nucleus, together with unchanged starting material (Fig. 1). The original phylloquinone was particularly abundant in the liver, which in turn contained no vitamin K_2. In all other organs, vitamin K_2 was found one hour after feeding, whereas only traces of vitamin K_1 were present (Fig. 2). The metabolism of the vitamin is obviously considerable, as the maximum concentration of labeled vitamin K_2 is reached after six hours. Thereafter the concentration di-

minishes and after one week only traces can be detected, mostly in the skeletal musculature.

These findings lead to the conclusion that only vitamin K_2 will be found in the body. Whether the K_2 vitamins with thirty and thirty-five carbon atoms in the side chain are taken up as such by the body cell or whether also in such compounds the side chain is exchanged against a geranyl-geranyl group, remains to be established.* The only organ in which one may expect to find phylloquinone itself, in all probability introduced through food uptake, is the liver. I consider it likely that in the liver vitamin K_1 supplants vitamin K_2 also functionally.

From the experimental explanation of these relations there arises the question of the mechanism by which the chemical transformations come about. The available experimental evidence stands to indicate that the phylloquinone side chain is split off as a unit. It could be traced in the extracts thanks to its C^{14} label and was found to occur in the form of a strongly lipophilic ester, which upon hydrolysis yields phytol.

Thanks to the work of Lynen,[4] Bloch and others we are well informed about the biosynthesis of polyisoprene chains by animal cells. It appeared plausible that the introduction of an isoprenic side chain in a quinone nucleus would take place by an analogous route as in the synthesis of the chain itself. In other words it may be assumed that the side chain is attached by elimination of pyrophosphate between the quinone (or the hydroquinone) and the pyrophosphoric acid ester of a polyisoprene alcohol (Fig. 3).

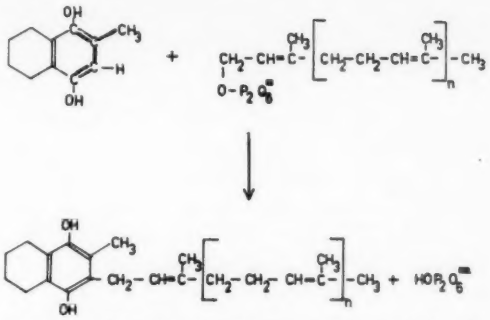

FIG. 3. Scheme for the introduction in methylnaphthohydroquinone of a polyisoprene side chain.

This hypothesis was easily confirmed by experiments in which labeled methylnaphthoquinone and pyrophosphates of polyisoprene alcohols of varying chain length were incu-

* Further experiments have shown that indeed in the rat and the chicken, vitamin $K_{2(20)}$ is converted into vitamin $K_{2(20)}$.

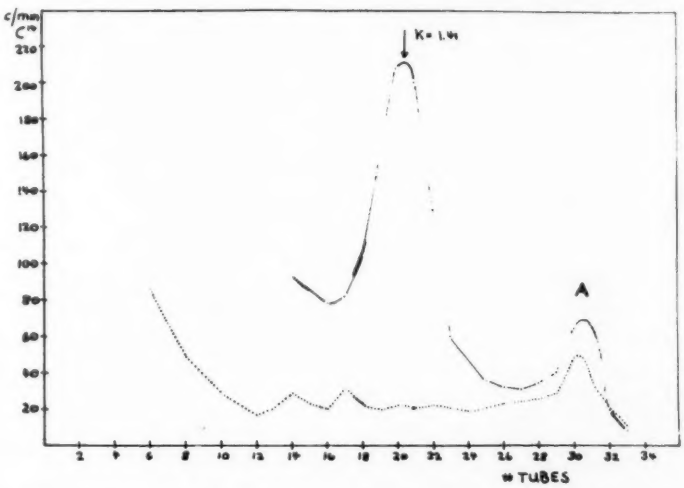

FIG. 4. CCD pattern of liver mitochondria lipid extract. Vitamin K deficient chicken. C^{14}-2-methylnaphthoquinone 2.5×10^{-4} M, geranyl-geranylpyrophosphate 10^{-3} M. System: n-neptane-methylglycol (.——. = lipid extract, ... = lipid extract control [without geranyl-geranylpyrophosphate]).

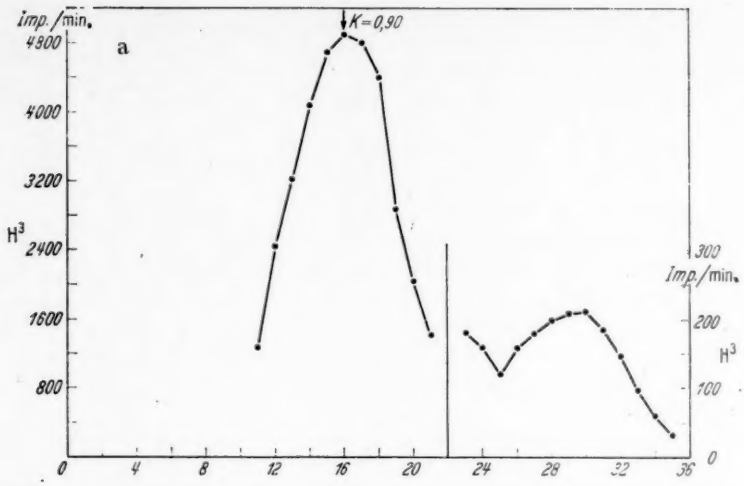

FIG. 5. Countercurrent distribution pattern of a rat liver homogenate lipid extract. H^3-5,6-dimethoxy-2-methylbenzoquinone 10^{-4}M, geranyl-geranyl-pyrophosphate 10^{-3}M. Formation of ubiquinone (20) and ubiquinone (45 and 50) (right curve).

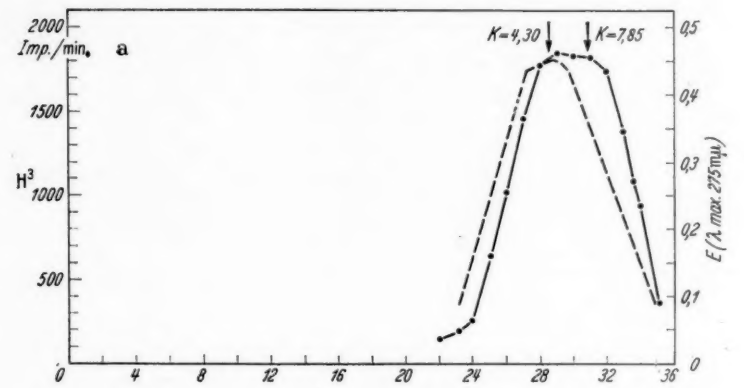

FIG. 6. Identical experiment as described in the previous figure but substituting geranyl-geranyl-pyrophosphate by solanesylpyrophosphate. Formation of ubiquinone (45) and (50). (.——. = radioactivity; --------- = extinction of authentic 5,6-dimethoxy-2-methyl-3-solanesylbenzoquinone).

bated with liver homogenates.[5] Examination of the mixtures after various periods in a countercurrent apparatus according to our usual technic led to the identification of vitamins of the K_2 group. Rat liver gives only low yields of the K_2 vitamins, whereas chicken liver again proved useful. The enzyme system responsible for the condensation is connected with the mitochondria fraction. No addition of microsome fraction or of cytoplasma is required. Disrupture of the mitochondria in a sonic oscillator sets the enzyme or enzyme system free: the supernatant liquid obtained by centrifugation in an ultracentrifuge contains a large fraction of the activity originally bound to the cellular particles. The enzyme does not have a high specificity with regard to the available pyrophosphate.

The alkylation was demonstrated with the pyrophosphates of geraniol, farnesol and geranyl-geraniol. There were thus obtained vitamin $K_{2(20)}$ (Fig. 4), the only product so far observed *in vivo*, and also the lower homologue with a C_{10} and a C_{15} side chain. We also made

the remarkable observation that phytol pyrophosphate does not react with methylnaphthoquinone under our experimental conditions.

In the course of our work we came across some results which led us to extend our study to ubiquinone (coenzyme Q).[5] In our incubation experiments with geranyl-geranyl pyrophosphate, for instance, we found beside geranyl-geranyl-naphthoquinone another labeled compound. It occurred only in small amounts and was considerably more lipophilic. In the distribution experiments it behaved as a K_2 vitamin with a side chain of forty-five or fifty carbon atoms. The same substance was formed also when no pyrophosphate of any of the side chain alcohols had been added. It followed therefore that the side chain can either be synthesized by or is present in the mitochondria. Isoprene chains of this length are present in ubiquinone, which is known to be formed in the body. We concluded that by adding labeled methylnaphthoquinone we had supplanted a natural unit of the synthesis of ubiquinone. We therefore repeated our experiments with labeled Q_0, that is with 2,3-dimethoxy-5-methyl-1,4-benzoquinone and found, as expected, that this compound reacted with all the polyisoprenyl pyrophosphates we added, yielding the corresponding ubiquinones with side chains of C_{10} to C_{45} (Figs. 5 and 6). The enzyme system of the liver mitochondria is considerably more active with dimethoxymethyl-benzoquinone as substrate than with menadione. The yields attain 25 per cent of the quinone. Mitochondria of rat liver are also found to be active.

We cannot as yet say whether the same enzyme is responsible for the formation of vitamin K_2 and of ubiquinone: some of our preliminary experiments point to identity of the enzyme, others are more easily interpreted by assuming that two different enzymes are at work.

We have extended our experiments to other quinones as well and found that toluquinone and trimethylbenzoquinone do not react, whereas 2-monomethoxy-5-methyl-1,4-benzoquinone is alkylated in position six by the enzyme.

These results indicate that the synthesis of ubiquinone in the body occurs along the same lines and perhaps even under the influence of the same enzyme as the formation of the "biological" K_2 vitamins from menadione or phylloquinone. The fundamental difference of the two processes is only that the quinone part in ubiquinone is also produced in the body by a mechanism that is not yet clearly understood, whereas in vitamin K_2 the cells depend on exogenous sources for the naphthoquinone nucleus, which they are unable to synthesize.

In my opinion, this close metabolic relationship of the two active principles finds its parallel in the more important functional one.

Several years ago, as you may know, I put forward the idea that vitamin K has nothing to do directly with the production of blood clotting proteins. It should act as an oxidation reduction catalyst in the phosphorylating respiratory chain along with other naturally occurring quinones. This hypothesis is based on the following experimental facts: (1) Liver mitochondria of highly K free chicken show a lowered P/O ratio compared with mitochondria from normal animals.[6] (2) This lowered capacity of oxydative phosphorylation can be restored *in vitro* by the addition of vitamin K_1 or K_2.[7] (3) All substances, which *in vivo* act as antagonists to vitamin K_1, the best known representative being dicumarol, act as very potent uncouplers of respiration and phosphorylation.[8,9]

According to its relatively negative ox/red potential the site of action of vitamin K in the respiratory chain should be between the pyridin nucleotides and cytochrome c.[10] We have tried for several years to detect an enzyme which could link the vitamin K with the pyridine nucleotides, a vitamin K reductase, and we were finally fortunate enough to isolate this enzyme in pure state.[11] It represents a so far unknown yellow enzyme with FAD as the prosthetic group. This latter can be reversibly split off and identified by paper chromatography or the d-amino acid oxydase test. The enzyme is reduced by the reduced forms of DPN and TPN as well. In the reduced state it can be reoxidized by a number of quinones, the most active being methyl naphthoquinon with a turn over number of 700,000. This high turnover number decreases if side chains are introduced in position three of the molecule. Vitamin $K_{2(20)}$ shows a turnover number of not more than 350 per minute. This

however may be due to the fact that in order to bring this water insoluble substance in solution one has to add detergents like Tween or others. Such substances seem to envelope and mask the quinon which as a consequence does not show its natural behavior which it may display when properly bound and fixed in the mitochondrial matrix.

Maybe the most interesting feature of this enzyme is the high rate of inhibition which one observes with dicumarol and all other anticoagulants. 10^{-8} M. dicumarol gives a ninety-three per cent inhibition and 10^{-10} M. dicumarol gives a 14 per cent inhibition of a 2×10^{-1} M. solution of the pure enzyme. This is to my knowledge the highest inhibition rate so far observed in any enzyme system. Thus, it seems to me very reasonable to assume that this high and specific inhibition of the enzyme, the mode of action of this enzyme itself as a hydrogen carrier and the known anticoagulant action of the inhibitors *in vivo* must have something to do with each other.

The enzyme vitamin K reductase is present in all kinds of animals, mammals, birds, fishes and amphibians. It is most abundant in the liver, kidney and heart muscle. When, however, pigeons were investigated it turned out that this species usually contains no detectable amount of vitamin K reductase and only in a few cases the reductase could be unambiguously determined.[12] Now it is interesting to note that pigeons in sharp contrast to other birds like chickens, geese or ducks are not dependent on a supply of vitamin K in the food. The question arises what consequence this lack of vitamin K reductase and apparent independence of vitamin K may have on the oxydative phosphorylation. According to my previously mentioned hypothesis, the vitamin K should be necessary for the first of the three phosphorylating steps during cell respiration. Using pigeon liver mitochondria we have investigated the P/O ratios which one gets with succinate and with β-hydroxybutarate as hydrogen donors. With succinate the normal value of about 2 (1.8) was observed; with β-hydroxybutyrate as substrate, however, we likewise obtained a P/O ratio of not more than 2.[1,9] This seems to me to indicate that in the pigeon liver only the energy potential between the succinate level and the oxygen is being used. Therefore, vitamin K and vitamin K reductase are not needed in this animal.

SUMMARY

Methylnaphthoquinone is converted in the animal organism into vitamin $K_{2(20)}$, which is the actual active form of the K vitamins. Vitamin K_1 (phylloquinone) as well as vitamin $K_{2(30)}$ (the K vitamin of bacteria) first lose the side chain and are thus converted into methylnaphthoquinone, which then yields vitamin $K_{2(20)}$. The side chain can be introduced *in vitro* in the presence of an enzyme system which occurs in liver mitochondria. The action of the enzyme system consists of condensing the pyrophosphates of isoprenalkohols with the quinone with elimination of pyrophosphoric acid and formation of the corresponding 3-substituted naphthoquinones.

It has been shown that the synthesis of the ubiquinones (coenzyme A) takes place along the same lines, starting from 2,3-dimethoxy-5-methyl-benzoquinone.

The properties of a new flavoprotein (vitamin K reductase) are described. This reductase transfers hydrogen from $DPNH^+$ and $TPNH^+$ to various quinones and is strongly inhibited by vitamin K antagonists (e.g., dicumarol). The importance of this enzyme for the interpretation of the mechanism of action of vitamin K is discussed.

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Possible Function of Vitamin K and Related Quinones in Oxidative Phosphorylation

R. DUNCAN DALLAM, PH.D.*

WE HAVE BEEN interested for some time now in the formation, transformation, utilization, distribution and control of energy at the subcellular level. During the past few years we have focused our attention on the energy reactions associated with rat liver mitochondria and more specifically those coupled with the oxidative processes utilizing the respiratory chain.

We have found that if one irradiates mitochondria with ultraviolet light the energetic efficiency; i.e., the amount of ATP formed per atom oxygen utilized, was lowered accompanying the oxidation of beta-hydroxybutyrate, whereas it was not altered when reduced cytochrome c was the substrate. It was further shown that added vitamin K_1 was able to restore the depressed system to near normal.[1,2] From these investigations it was concluded that (1) vitamin K_1 was either directly or indirectly concerned with the phosphorylative mechanism accompanying oxidation and (2) the phosphorylative site(s) affected was between DPN^+ and cytochrome c.

Recently we have extended these irradiation studies and attempted to correlate the irradiation effect with certain fairly well established energetic reactions that occur in mitochondria. In Figure 1 we see that phosphorylation associated with oxidation is inhibited by irradiation with ultraviolet light to a lesser extent than is the P_i-ATP exchange reaction. This indicated, as has been suggested by others, that the limiting reaction in the sequence of reactions concerned with the phosphorylative processes is the initial set of reactions concerned with the transfer of energy to some intermediate compound; i.e., steps 1 and 2, Figure 2. In preliminary comparative experiments it has also been found that DNP stimulated ATPase is inhibited to a lesser degree while magnesium stimulated ATPase to a greater degree than either oxidative phosphorylation or P_i-ATP exchange. These recent findings appear to have certain implications regarding the over-all question of phosphorylative reactions related to oxidation but will not be elaborated.

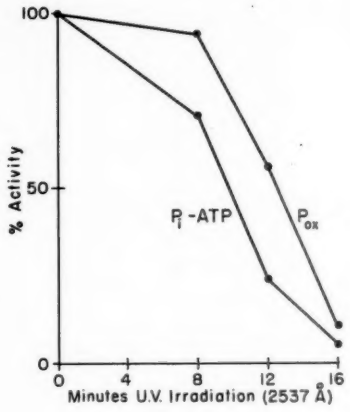

FIG. 1.

1) $AH_2 + X \rightleftharpoons XH_2 + A$
2) $XH_2 + B \rightleftharpoons \sim X \sim + BH_2$
3) $\sim X \sim + Pi \rightleftharpoons X \sim P$
4) $X \sim P + ADP \rightleftharpoons ATP + X$

FIG. 2.

Let us now turn our attention to the question of how vitamin K_1 could participate in these

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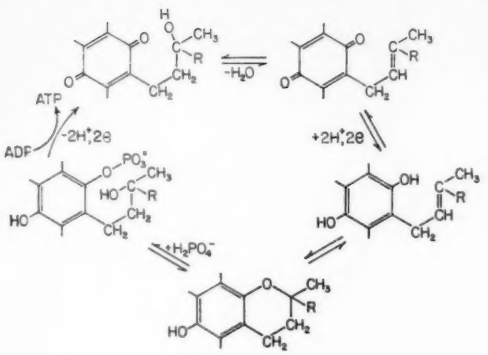

FIG. 3

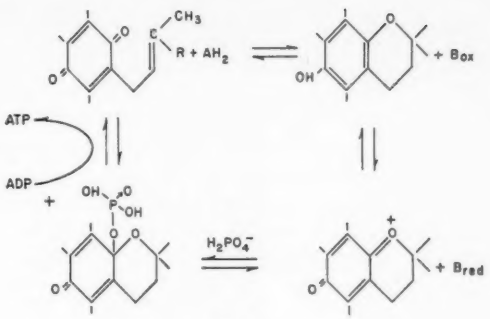

FIG. 4

coupled energy reactions. Several other investigators have speculated that vitamin K and quinones, in general, could act as a cofactor in the production of ATP by oxidation of quinol phosphate in the presence of a suitable acceptor such as ADP. Professor Taylor and I approached this phase of our work by first considering the problem of how inorganic phosphate could combine with quinones to form a phosphoquinol, a step which must necessarily precede the oxidation-energy transfer reaction.[3]

Examination of the structure of several quinones which have been implicated in biological oxidation and to a lesser extent in the accompanying phosphorylations, vitamin K, ubiquinone and vitamin E, reveals several common features; all are para quinones; all are completely substituted, and all possess a side chain with basically the same carbon skeleton. Although two of these, vitamin K_1 and ubiquinone possess unsaturated open side chain and vitamin E is a ring structure, it is well known that the two side chain structures, open and ring, are readily interconvertible chemically. For example, the quinone molecules with an open isoprenoid side chain can be reduced to their respective hydroquinones but if this is carried out in acid solution one obtains the cyclic derivatives of the tocopherol type. It should be emphasized that these three compounds are able to cyclize during reduction to form the tocopherol derivative. Thus, we see that these three quinones have similar structures and are capable of undergoing similar chemical transformations. May these similar chemical reactions in any way afford a pathway for the incorporation and transfer of energized inorganic phosphate to ADP during mitochondrial oxidative phosphorylation?

Considerations of the energetics of any proposed mechanism absolutely require the introduction of inorganic phosphate into the hypothetical intermediate in some manner which does not require a great deal of energy; i.e., there must be an equilibrium between inorganic phosphate and the organic phosphate which is not too far to either side and, of course, ATP cannot be used up. Once this can be done, the possibilities of oxidation of the hydroquinone phosphate to a high energy compound presumably could follow the reaction pathway suggested by others.

One such hypothetical mechanism proposed by us is seen in Figure 3. Here we see that a quinone with the proper prerequisites, such as vitamin K, ubiquinone or trimethyl-phytyl benzoquinone (oxidized form of vitamin E) can undergo reduction to the quinol and then cyclize to the tocopherol. Inorganic phosphate might then be capable of splitting the chroman ring by phosphorolysis resulting in the formation of the hydroquinone monophosphate ester with a hydroxylated side chain. Attention should be focused on a primary requisite of this and other schemes we have proposed; i.e., cyclization of the side chain to form a chroman ring precedes inorganic phosphate combination with the quinone. Preliminary experiments have indicated that cyclization of the quinone is probably an essential in the functioning of vitamin K in the irradiated systems. When the beta-gamma dihydrophtyl derivative of vitamin K_1,

TABLE I
Effect of Benzoquinones on Oxidative Phosphorylation

Compound	Molar Concentration	P_i (μM)		O (μatoms)		Activity* (%)	
		Control	Quinone	Control	Quinone	Phos.	Ox.
U_0	$1x10^{-3}$	3.31	0	2.64	0	0	0
	$2.2x10^{-4}$	2.91	0	2.45	0	0	0
	$5x10^{-5}$	3.32	2.23	2.90	2.42	80	83
	$1x10^{-5}$	3.32	3.03	2.80	2.65	93	95
U_5	$1x10^{-3}$	4.11	1.52	3.17	2.90	37	93
	$1x10^{-4}$	5.00	4.50	3.36	3.20	90	96
	$1x10^{-5}$	4.00	3.80	3.50	3.40	95	96
Trimethyl phytyl quinone	$1x10^{-3}$	3.39	1.25	2.39	1.44	40	81
	$1x10^{-5}$	3.90	3.63	3.01	2.81	93	93
U_{phytyl}	$2x10^{-3}$	5.14	4.40	2.18	2.15	88	100
	$1x10^{-3}$	6.01	5.71	2.59	2.59	95	100
	$2x10^{-4}$	4.00	3.83	2.30	2.30	96	100
	$1x10^{-5}$	5.00	5.00	3.00	3.00	100	100
U_{50}	$1x10^{-4}$	3.52	3.41	2.71	2.70	96	100
	$1x10^{-5}$	2.96	2.96	2.60	2.60	100	100

* Per cent inorganic phosphate exchanged of control.

which cannot cyclize due to its saturated side chain, was added to irradiated mitochondria, no energetic restoration was observed.[3] According to Clark et al.,[4] the phosphoquinol could in the presence of ADP be oxidized to yield ATP and the starting quinone.

In our hypothetical mechanism and those of others the final reaction involves the oxidation of the quinol phosphate in the presence of a suitable acceptor, presumably ADP, to yield ATP and the original quinone. However, it would appear that all of these mechanisms require revision due to a discrepancy between these proposals and the existing data. The fact that P_i-ATP exchange occurs extremely rapid and equally well either in the presence or absence of substrate, as originally shown by Boyer et al.,[5] almost demands that the intermediate compound, X∼P does not undergo oxidation for exchange and thus would not require oxidation as one of the final steps in the generation of ATP (see Fig. 2). It would thus appear that the previously proposed mechanisms are incorrect. In Figure 4, a simplified mechanism is presented that perhaps more accurately depicts the chemical mechanisms concerned with mitochondrial oxidative phosphorylation if quinones do indeed play a direct role in ATP formation.

The starting quinone can be any fully substituted paraquinone possessing an isoprenoid side chain adjacent to one of the quinoid groups; i.e., vitamin K, ubiquinone and the group of oxidized E vitamins. Such a quinone could be reduced, presumably by one of the members of the respiratory chain (AH_2) and then oxidized presumably by another member of the chain (B_{ox}) to yield the oxonium derivative which would freely resonate to the carbonium ion. This electrophilic compound, presumably stabilized by the enzyme, in the presence of nucleophilic inorganic phosphate, could be converted to the phosphorylated hemi-ketal which then could donate the energized phosphate to ADP under proper conditions. The validity of the above proposal is currently being investigated through the aid of synthetic compounds and for the present, this scheme is accepted as a working hypothesis. A similar scheme has been proposed by others.[6,7]

Since we have been thinking about general reactions of quinones; i.e., reactions which not only apply to vitamin K but also ubiquinone, the possible role of ubiquinone in oxidative phosphorylation has also been investigated.[8] Intact rat mitochondria have been used which retained the internal ubi-

TABLE II
Effect of Benzoquinones on P_i^{32}-ATP Exchange Reaction

Compound	Molar Concentration	P_i Exchanged (μM.)		Activity (%)
		Normal	Quinone	
U_0	2.17×10^{-3}	0.90	0.008	0
	2.17×10^{-4}	0.90	0.008	0
	1.1×10^{-4}	1.34	0.046	4
	2.17×10^{-5}	0.90	0.65	70
	2.17×10^{-6}	0.90	0.82	91
U_5	2.17×10^{-3}	0.90	0.008	0
	1.1×10^{-3}	1.34	0.28	21
	2.17×10^{-4}	0.90	0.54	60
	5×10^{-5}	0.90	0.62	69
	2.17×10^{-5}	0.90	0.73	78
	2.17×10^{-6}	0.90	0.83	92
U_{phytyl}	2.17×10^{-3}	0.90	0.76	85
	2.17×10^{-4}	0.90	0.76	85
	2.17×10^{-5}	0.90	0.76	85
	2.17×10^{-6}	0.90	0.81	92
U_{50}	2.17×10^{-4}	1.34	1.20	90
	2.17×10^{-5}	1.34	1.20	90
	2.17×10^{-6}	1.34	1.29	92

quinone presumably intact. Varying concentrations of several homologues of ubiquinone were added separately and in combination to mitochondrial systems undergoing energy transformation and to systems carrying out P_i-ATP exchange. In Table I see that only ubiquinone$_0$ and trimethyl phytyl benzoquinone inhibit oxidation. Because of the greater amount of inhibition of ubiquinone$_0$ it would appear that the absence of a side chain on the quinone has a greater effect on mitochondrial oxidation than the substitution of methyl for methoxy groups. However, since phosphorylation was completely inhibited by ubiquinone$_0$ at this concentration, this conclusion is presented with reservations. It is also seen that ubiquinone$_0$, ubiquinone$_5$, trimethyl phytyl benzoquinone and ubiquinone-phytyl, in high concentration, inhibit phosphorylation associated with oxidation and the magnitude of inhibition is in that order.

These data suggest that the added ubiquinone homologues that inhibit the phosphorylative process may be acting as competitive inhibitors; i.e., by competing with the internal ubiquinone and for specific enzymes. Also, the decrease of inhibitory effect with increase in length of the isoprenoid side chain seems to indicate that the isoprenoid side chain is necessary for the proper function of ubiquinone.

In Table II we see that the P_i-ATP exchange reaction is inhibited by ubiquinone$_0$ and ubiquinone$_5$ but not substantially by ubiquinone$_{phytyl}$ or ubiquinone$_{50}$ and that this inhibition, like that observed with oxidative phosphorylation, is concentration dependent. In Figure 5 the graphic presentation of the data from Table I and II is shown.

In Table III it is seen that partial reversal of the inhibition is achieved when ubiquinone$_{phytyl}$ or ubiquinone$_{50}$ are added to the ubiquinone$_0$ and ubiquinone$_5$ inhibited P_i-ATP exchange systems. The restoration can be seen more clearly in Figure 6 which is a graphic reproduction of the data presented in Table III.

TABLE III
The Effect of U_{phytyl} or U_{50} on U_0 or U_5 Inhibited P_i-ATP Exchange

Molar Concentration Quinone(s)*	Activity† (%)					
	U_0	$U_0 + U_p$	$U_0 + U_{50}$	U_5	$U_5 + U_p$	$U_5 + U_{50}$
1.1×10^{-3}	...	...	...	21	44.5	...
2.17×10^{-4}	0	7	0	60	80	70
2.17×10^{-4}	4	17	14[a]	...	...	...
5×10^{-5}	...	...	...	69	86[b]‡	86[a]§
2.17×10^{-5}	70	74[b]	74[a]	...	...	...

* In all cases, the concentration of U_p or U_{50} added is the same as that of U_0 or U_5 unless otherwise specified.
† Percent inorganic phosphate exchanged of control.
§ a=U_{50}, 1×10^{-4} M.
‡ b=U_p, 1×10^{-4} M.

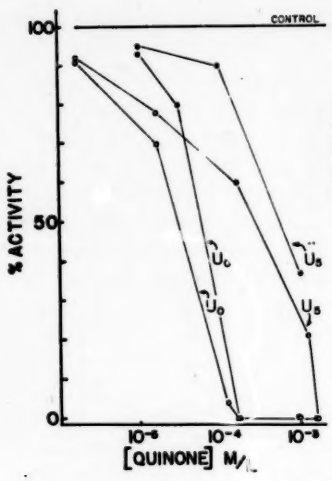

FIG. 5

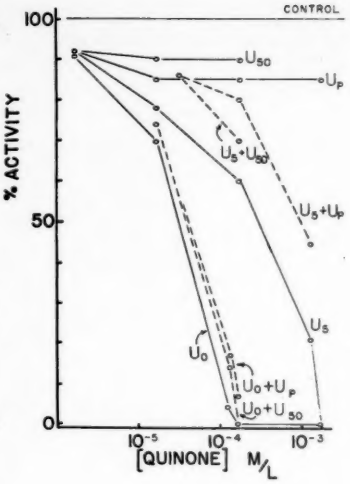

FIG. 6

SUMMARY

In conclusion, from the data presented it would appear that vitamin K and ubiquinone may participate in the transformation of energy during mitochondrial oxidation reduction reactions. Whether they are acting as cofactors and enter into the chemical reactions directly is a question that cannot yet be answered, at least, not by this investigator.

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Interrelationships Between Vitamin K and Estrogenic Hormones

SUSAN J. MELLETTE, M.D.*

IMPETUS for the present study was provided, rather dramatically, by an observation made nearly three years ago here in the laboratories of Dr. B. Connor Johnson. Hemorrhagic deaths began occurring in large numbers in a group of rats being fed a diet containing cooked ground beef which had been sterilized by irradiation, flour, sucrose, salts and a vitamin mix which did not contain vitamin K. The somewhat remarkable aspect of this phenomenon as initially observed was that only male rats seemed to be susceptible. Prevention or cure of the hemorrhagic state by vitamin K was readily demonstrated.[1]

In spite of scattered reports in the literature indicating occasional spontaneous hypoprothrombinemia in rat colonies, the concept that the rat does not require supplemental vitamin K had been widely accepted. Recent studies indicate that a dietary requirement does indeed exist, particularly under certain conditions. A partial explanation, at least, may be that coprophagy is practiced to a varying extent by animals fed diets of differing composition. Diets of the type mentioned, containing irradiated beef, must be considered as readily conducive to the development of a vitamin K deficiency: but repeated studies in several laboratories have indicated that the irradiation alone is not responsible and that similar results may be obtained with a variety of diets. B. E. Gustaffson, in work presented at the Fifth International Vitamin Symposium (September 1–7, 1960, Washington, D. C.), further reports that male rats develop vitamin K deficiency more readily than female rats when the animals are maintained under germ-free conditions.

PROTECTIVE EFFECTS OF ESTROGENS IN DIETARY VITAMIN K DEFICIENCY IN THE RAT

Studies aimed at a determination of mechanisms involved in hemorrhagic diatheses in the rat were begun in our laboratory during the summer of 1958. Except as otherwise indicated all results to be reported here were obtained with the Charles River CD rat. Plasma prothrombin concentration was determined by the one stage viper venom method of Hjort, Rapaport and Owren.[2] Other experimental details were essentially as previously reported.[3] Initial studies involved castrate as well as intact animals of both sexes fed diets similar in composition to those used by Metta and Johnson and containing either irradiated or untreated cooked ground beef. Mortality during a twelve week feeding period in these animals is indicated in Table 1. Deaths occurred earliest in intact males but castrate female rats were al-

TABLE 1
Hemorrhagic Deaths During Twelve Weeks of a Vitamin K Deficient (Irradiated Beef) Diet

Subjects	No. of Rats	No. of Deaths	Average Survival Time (days)
Male rats			
Intact	10	10	17
Castrate	10	10	40
Female rats			
Intact	10	3	33
Castrate	10	10	29

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* Assistant Research Professor in Internal Medicine and Co-Director, Division of Cancer Studies.

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Presented at the Symposium on the Metabolism and Function of the Fat-Soluble Vitamins A, E and K on November 7 and 8, 1960, at the University of Illinois, Urbana, Illinois, under the sponsorship of The National Vitamin Foundation, Inc., New York. Now York.

TABLE II

Sex Differences During Growth in the Prothrombin Level of Rats Fed a Stock Laboratory Diet*

Age (days)	Average Daily Gain for Each Sex (gm.)		No. of Determinations		Rats with Prothrombin Level of 100 Per cent or More (%)	
	Male	Female	Male	Female	Male	Female
24 to 30	4.4	3.9	10	9	80	67
31 to 60	5.7	4.0	57	52	54	85
61 to 90	3.0	1.5	54	29	54	93
91 to 120	1.7	0.7	27	23	22	100
121 to 150	1.7	0.6	26	7	65	100
151 to 200	...	...	11	11	91	100
201 to 250	...	...	11	9	91	100

* Rats used were of The Charles River CD Strain. The diet consisted of Purina dog chow.

TABLE III

Effect of Estrogen Administration in Adult Male Rats Receiving a Vitamin K Deficient Diet

Group of Animals	Average Weight (gm.)		Average Prothrombin (%)		Cumulative Mortality (%)
	Day 0*	Day 3	Day 0*	Day 3	Day 18
Control group	410	424	77	12	100
Estrogen-treated group†	419	404	73	28	40

* Irradiated beef diet begun on Day 0.

† Estradiol sodium benzoate 50 μg. on Day 0 and at three day intervals through Day 15.

most as susceptible to hemorrhage. Transitory but definite protection was afforded by castration in the male animal. In comparable groups fed diets containing nonirradiated beef two deaths due to hemorrhage occurred among the ten intact male animals.

Marked differences in the prothrombin levels of the various groups were found during the feeding period.[3] Of particular interest were the consistently low prothrombin values (13 to 23 per cent) found in all initial determinations in samples obtained from intact male animals receiving the nonirradiated beef diet. Clearly, irradiation of the beef in these particular diets was not necessary for the production of marked hypoprothrombinemia. Castrate animals demonstrated moderately low prothrombin levels under these conditions, but intact female rats never appeared to be vitamin K deficient. Spontaneous increases in prothrombin occurred in all groups except the intact males fed the irradiated diet. This improvement was transitory in the castrates but was maintained in the intact female subjects in spite of continued feeding of the deficient ration. The marked resistance of the female rat to the development of vitamin K deficiency has also been demonstrated in animals fed a synthetic vitamin K-low diet containing casein as the source of protein. Prevention of coprophagy in such animals increased susceptibility to the diets but to a limited degree in contrast to the results in male rats.[4]

We have performed serial determinations of prothrombin in male and female rats from weaning to maturity during maintenance on a stock commercial diet (Purina dog checkers). Results are summarized in Table II which indicates the proportion of animals in each age group which maintained a prothrombin level at or above the "standard value" of 100 per cent. This value and the calibration curve for our studies were originally derived from pooled samples from adult rats of both sexes of the same strain. The differences apparent between male and female rats at the time intervals studied are roughly consistent with the development of sex hormone secretion. In spite of many attempts, including paired feeding by us and others, starvation experiments and similar approaches, we cannot consistently relate the greater rate of growth of the male rat directly to the phenomena observed. It should be noted, parenthetically, that the particular stock diet fed in the study just described has been discontinued as a laboratory diet by the manufacturer. Comparative studies indicate that prothrombin levels of male rats are considerably higher with the Laboratory Chow.

It is, however, pertinent to emphasize that

TABLE IV

Prothrombin Time Determinations for Vitamin K Deficient Male Rats Twenty Hours Following a Single Injection of Menadione or Estradiol*

Treatment	Determinations of Prothrombin Time (seconds)
Experiment I	
No treatment	47, 120, 120, 120, 120
Menadione (40 γ)	16, 21, 22, 21, 17, 17
Estradiol (1 mg.)	20, 17, 20, 120, 120
Experiment II	
No treatment	120, 120, 120, 120, 120
Estradiol (1 mg.)	19, 28, 15, 18, 19, 37

* Unpublished data obtained from B. C. Johnson.

hypoprothrombinemia may be present, in male rats particularly, at certain stages of development without any effort having been made to induce vitamin K deficiency. Unless coagulation studies are performed or the animals develop gross hemorrhage, this particular deficiency state may be overlooked. Even in animals which eventually survive vitamin K deficient diets, slight bloody coryza or some hemorrhage around the eyes is almost invariably noted. In animals begun on diet at about five weeks of age, these symptoms are most pronounced at about the eighth day of feeding and may occur transiently in female as well as in male rats.

It would further be expected that animals would differ in their susceptibility to diets severely deficient in vitamin K in a predictable manner dependent upon the sex of the rat and the stage of maturity at which such a diet was begun. Such an effect was demonstrated in experiments in which irradiated beef diets were begun at several ages.[3] After one month of feeding, mortality was about 40 per cent in males and 12 per cent in female rats begun at five weeks of age or less. When, however, animals were first started on the deficient diets after twelve weeks of age, the mortality in male rats increased to nearly 90 per cent but no further deaths occurred among the female animals.

The next obvious approach to the problem was to determine the effects of hormone administration. Even in adult intact male rats fed the deficient irradiated beef diet, injections of estradiol sodium benzoate* resulted in a considerable degree of protection against hemorrhage and in higher levels of plasma prothrombin. The results of one experiment are shown in Table III. Such a protective effect of estrogen administration has been very recently confirmed by B. C. Johnson in experiments in which blood samples were obtained twenty hours after the injection of menadione or estradiol in vitamin K-deficient male rats (Table IV).

Conversely, we have found that the suscep-

* The estradiol sodium benzoate, estradiol, and testosterone propionate used in these studies were generously supplied by the Schering Corporation.

TABLE V

Effect of a Single Large Dose of Warfarin Sodium Administered Parenterally to Male and Female Rats

Group and Sex*	Age (days)	Warfarin (mg.)	Prothrombin Concentration† (%)		Cumulative Mortality (%)	
			Mean	Range	Day 1	Day 5
Group A						
Male rats	35	1.0	<6	4 to 11‡	85	100
Female rats	35	1.0	<6	4 to 12	17	67
Group B						
Male rats	35	0.0	95	83 to 103	0	0
Male rats	54	1.0	15	9 to 19	33	100
Female rats	35	0.0	109	107 to 113	0	0
Female rats	54	1.0	13	8 to 19	17	33

* There were six rats of each sex per group.
† Determinations were made eighteen hours after administration of warfarin sodium.
‡ Prothrombin concentration of 4 per cent indicates lower limit of method as used, i.e., clotting time of greater than 80 seconds, 1:10 dilution.

TABLE VI

Pretreatment with Estradiol in Adult Male Rats Receiving a Single Injection of Warfarin Sodium

Treatment*	Prothrombin Concentration† (%)		Mortality (%)
	Mean	Range	
Sesame oil	18	15 to 19	60
Estradiol sodium benzoate (50 μg. in sesame oil)	45	27 to 60	20

* The treatment was daily for two days prior to administration of the anticoagulant.

† Determinations were performed eighteen hours after a single injection of 1.5 mg. warfarin sodium.

TABLE VII

Administration of Estrogens at Same Time as Anticoagulant: Effect on Prothrombin Levels and Mortality of Five Week Old Male Rats

Treatment	No. of Rats	Prothrombin Concentration* (%)		Mortality (%)
		Mean	Range	
Warfarin sodium (1 mg. subcutaneously)	6	24	20 to 30	67
Warfarin sodium plus conjugated estrogens† (0.2 mg. intramuscularly)	6	52	30 to 70	33

* Determinations were performed six hours after treatment.

† Premarin.

tibility of the adult female to the development of K deficiency can be increased somewhat by repeated administration of androgens. The dose of testosterone propionate used, however did not significantly alter prothrombin levels or mortality in female rats of the same age on less deficient diets (nonirradiated beef or Purina dog chow). The results obtained by Malhotra and Reber in experiments in which sex hormones were administered to intact or castrate male rats also indicate that mortality during irradiated beef feeding was greater in animals in which testosterone pellets had been implanted.[5]

EFFECT OF SEX HORMONES IN RATS RECEIVING A SINGLE LARGE DOSE OF AN ANTICOAGULANT

Although the observations just reported indicate a protective effect of female hormones in dietary vitamin K deficiency, it does not necessarily follow that such effects occur in hypoprothrombinemia induced by other means. We felt that it would be of interest to evaluate possible sex differences in the rat in response to an anticoagulant of the coumarin type. Warfarin sodium, 3-(*a*-acetonyl benzyl)-4-hydroxy coumarin (Coumadin®, Endo Laboratories), was selected because of its rapid action and the fact that it may be readily administered parenterally.

In an initial experiment (Table v), five week old male and female rats received a large (1 mg.) dose of warfarin sodium. Coagulation factors were determined in blood samples obtained by cardiac puncture eighteen hours after the anticoagulant. The sex difference in mortality is much more striking than the prothrombin determinations, all of which were quite low. It must be recognized, however, that the effect of the anticoagulant is always superimposed upon the pre-existing vitamin K status of the animal. The data for control animals (Group B, thirty-five days old) in the experiment shown in Table v indicates that, as expected, prothrombin values in male rats were already slightly less than those of the female rats. When, however, these animals later received warfarin, the post-treatment prothrombin values were essentially the same in the two groups. In spite of this, mortality in the male group was twice that of the female group.

Several other experiments of this sort have been performed in which the preliminary and postanticoagulant prothrombin levels of individual animals could be compared. When survival alone is considered, correlation is better between the sex of the animal and resistance to the anticoagulant than between such resistance and the actual prothrombin level as determined at a given time or the quantitative depression secondary to the anticoagulant.

Studies have also been made of the effects of exogenous hormones in conjunction with anticoagulant administration. These include ex-

periments in which estrogens were administered prior to, at the same time, or following the anticoagulant. In Table VI, results are shown for a group of older male rats (eight months old) which had been treated with estradiol sodium benzoate for two days before warfarin was administered. Prothrombin levels were higher and mortality considerably less in the estrogen-treated animals. Conversely, in a single experiment, mortality following anticoagulant in female rats was increased by pretreatment with testosterone propionate.

A more stringent test was introduced by evaluating the effect of estrogens given to animals which had received the anticoagulant some hours previously. For these studies, a preparation of water soluble conjugated equine estrogens (Premarin®*) was employed. This material has had considerable clinical use in nonspecific hemorrhagic conditions; for example, in epistaxis and in certain types of postoperative bleeding. The intravenous injection of this preparation into dogs was found by Johnson[6] to increase prothrombin and Factor V levels for a period of about three hours. In our study of the rat, blood sampling was performed at a time two hours after the intraperitoneal injection of 0.2 mg. of conjugated estrogens and eighteen hours after the administration of 0.5 mg. warfarin sodium. Prothrombin values were low (all below 20 per cent) in all groups, being slightly higher in those receiving the estrogens. In contrast to Johnson's results in the dog, we have not found significant changes in Factor V following administration of estrogen to rats, although this clotting factor has been followed in a number of experiments. This observation is particularly mentioned since deficiencies of Factor V may affect the results of one stage tests of "prothrombin time" of the Quick type, potentially leading to confusion in the interpretation of studies aimed primarily at vitamin K dependent coagulation factors.

In the animals pretreated with warfarin, mortality was somewhat lower in the male group also receiving conjugated estrogens. Three of the ten rats in this group succumbed compared to five of the ten animals receiving only the anticoagulant. A 20 per cent mortality was found in both female groups. In a repeat experiment, using small groups of older animals and a still lower dose of warfarin, there was no evidence of a protective effect of the conjugated estrogens under the conditions employed.

An apparently definite effect of the estrogens is, however, evident if the anticoagulant and the conjugated estrogens are given at approximately the same time and blood samples obtained after six hours. Such an experiment is illustrated in Table VII.

In spite of all the evidence presented for the participation of the female sex hormones in the modification of vitamin K deficiency states, there may well be genetic as well as hormonal differences between the two sexes which contribute to some of the results observed. Certainly the sex hormone secretion is minimal in extremely young rats, yet a marked difference in response to a single dose of warfarin sodium occurred in male and female rats only two days postweaning. Mortality of the male sex was 100 per cent within two days whereas, that of the female sex was 40 per cent at the end of a two week observation period. Such differences are, perhaps, related to other well accepted similar phenomena, even including the somewhat higher perinatal mortality of the human male infant.

HORMONAL ACTIVITIES OF THE K VITAMINS

We next turned our attentions to the other side of the coin. Could estrogenic activity be demonstrated for the K vitamins? Some evidence was obtained from the literature. In Professor Dam's review on vitamin K in the *Annual Review of Biochemistry* in 1951, reference was made to the work of Chamorro,[7] who reported increases in the uterine weights of prepubertal rabbits secondary to the administration of menadione, and, immediately following, to two studies published in the Italian literature. The reports of estrogenic activity were sandwiched among multiple other reported activities of vitamin K ranging from its efficacy in preventing dental caries to its value in the treatment of hives and of whooping cough.

Our studies on this particular problem are just well under way and the material presented

* Premarin was generously supplied by Ayerst Laboratories.

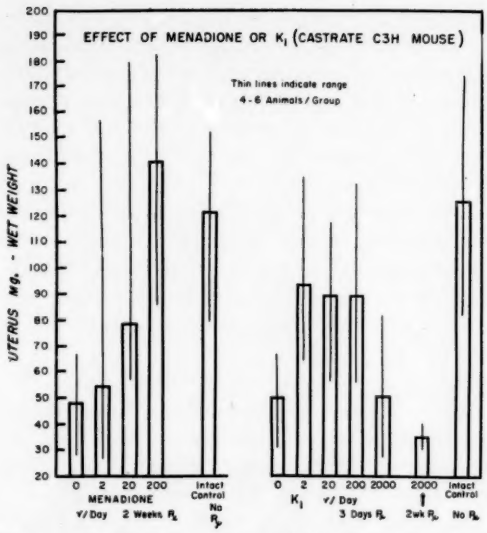

FIG. 1. Changes in uterine weights of castrate mice occurring with the parenteral administration of K vitamins.

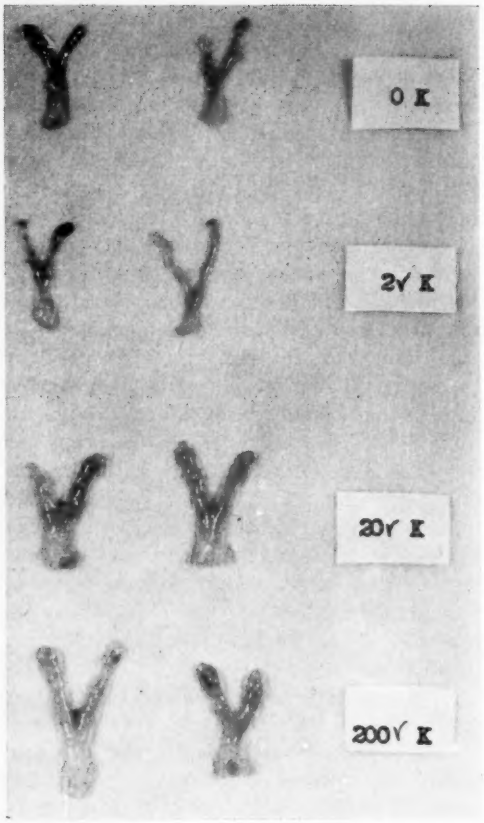

FIG. 2. Uteri from castrate mice treated with menadione for a two week period at the daily dose indicated.

is included because of its interesting aspects rather than as a completed project.

An increase in the uterine weight of the castrate mouse following the parenteral administration of either menadione or vitamin K_1 was readily demonstrated. Figure 1 illustrates the increasing weights obtained with increasing amounts of menadione given five times weekly for a two week period. A photograph of representative uteri from this experiment indicates the turgid, hypertrophied and glistening uteri of the treated animals at the higher levels of menadione in contrast to the untreated and 2 μg. levels (Fig. 2).

The data illustrated for treatment with K_1, also in Figure 1, should not be directly compared with that for menadione since the experimental period was only three days at comparable dose levels. As shown in this diagram (and also in Fig. 3) decreasing effects on uterine weight are found either with increasing dosage levels of K_1 or prolonged administration. This result has been confirmed in several other experiments. Daily administration of a 1 μg. dose in another experiment yielded progressive increases in uterine weights at twenty-four, and at seventy-two hours. At the latter time, the average weight was more than twice that of the castrate controls. A moderate further increase was recorded after one week: but, by the end of two weeks, the uteri were approximately the same size as those noted at twenty-four hours. Such paradoxical effects are certainly not unexpected with various hormones.

The ability of vitamin K_1 to decrease the uterine weight response to estradiol rather than to yield a summative effect is illustrated in Figure 3. In this experiment the injections either of K_1 or estradiol, or both, were given five times weekly for two weeks. Different sites were used for the two materials in mice receiving both. The average weights of the uteri from animals receiving estradiol were in the 100 mg. range (lowest 73 mg.) compared to an average of 75 mg. (range 57 to 85 mg.) when 5 μg. of K_1 was administered along with the estradiol. One atrophic uterus persisted in a K_1 treated animal receiving the 5 μg.

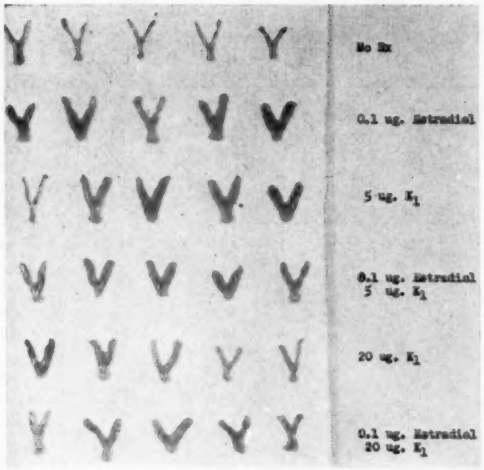

FIG. 3. Uteri from castrate mice receiving estradiol and/or vitamin K_1. (See text.)

dose, but weights of the other uteri from animals in this group were from 70 to 120 mg. This experiment also again illustrates the decreasing effectiveness of higher dose levels of K_1, the average uterine weight in the 20 μg. K_1 group being only 49 mg. Suppression of the expected estradiol effect by K_1 is also evident at the 20 μg. level. Suppressive rather than summative effects occurring when two estrogenic substances are administered simultaneously have been reported with estriol and certain other members of a group designated the "impeded estrogens."[8] Our results with K_1 are suggestive of similar effects.

Additional studies indicate that the castrate or immature rat, as well as the mouse, responds in the manner just described. Cornification of the vaginal epithelium, typical of an estrogenic effect, occurs in both rats and mice treated with the K vitamins administered parenterally in the doses mentioned.

SUMMARY

Evidence has been presented indicating a protective effect of estrogenic hormones in vitamin K deficiency. Female rats are more resistant to the development of dietary K deficiency than are male animals. This difference is most pronounced during early adulthood. Castration increases the susceptibility of the female rat and decreases that of the male rat to hypoprothrombinemia and hemorrhagic death during the feeding of vitamin K-deficient diets. In male animals fed similar diets, the injection of estrogens results in improved prothrombin levels and a lower mortality. Administration of testosterone has an opposite effect.

A single large dose of the anticoagulant, warfarin sodium, is followed by a greater mortality due to hemorrhage in male rats than in female rats of the same age. In male animals some protection against the effects of the anticoagulant is afforded by pretreatment or by simultaneous administration of estrogenic substances.

Effects considered to be characteristic of estrogenic hormones, for example, increases in the uterine weight and cornification of the vaginal epithelium in castrates, have been shown to follow the administration of both K_1 and menadione. With K_1, at least, paradoxical effects occur with increasing dose level or continued administration. When K_1 is administered concomitantly with estradiol, uterine weights are considerably decreased in comparison to those resulting from the same dose of estradiol given alone.

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DISCUSSION

DR. PAUL GRIMINGER (*New Brunswick, New Jersey*): It is most interesting to note that, with chicks, we have not been able to observe the sex difference in vitamin K deficiency described by Dr. Mellette. In fact, we have been using female chicks frequently for vitamin K assay work and the evaluation of different forms of vitamin K. However, we have had difficulty in obtaining severe vitamin K deficiency in the laying hen. Since we have never done any work in this area with the adult

TABLE I
Blood Coagulation Times of Adult Chickens*

Sex	Dates Determinations Were Performed			
	January 1959	June 1959	January 1960	June 1960
Male chickens	3.8	4.6	10.0	8.0
Female chickens	6.3	8.3	14.1	12.2

* Average in minutes of several hundred chickens on various dietary treatments.

male chicken, I cannot say at the present time whether this has any connection with estrogenic activity. Dr. Mellette's talk has certainly given me cause to consider this possibility, and I am planning to test it experimentally as soon as we can design an appropriate experiment. We have, however, obtained a response to graded levels of Dicumarol® in the laying hen, which could be counteracted with vitamin K_1. Measuring prothrombin and coagulation times of several hundred adult male and female chickens over a period of eighteen months, we found no differences in prothrombin times, but observed longer coagulation values for the females at every sampling (Table I). In our experiments, K_1 counteracted Dicumarol in the growing chicken completely, while K_3 failed to do so (Table II). It is interesting at this point to speculate on the possibility of an involvement of an estrogenic activity of K_1.

I would also like to make a few remarks with regard to the statistical treatment of blood coagulation and prothrombin data. Since neither prothrombin nor coagulation times are normally distributed, it is incorrect to use statistical methods designed for populations following, or at least approaching, a normal or Gaussian distribution, as signified by the symmetrical bell-shaped curve. It is possible, however, to transform the data, and then apply conventional statistical methods. For blood coagulation times, we are using

TABLE II
Plasma Prothrombin Times of Chicks Receiving Dicumarol*

Supplement	Level (mg./kg. diet)	Vitamin K_1 Equivalent (mg./kg. diet)	Prothrombin Time† (seconds)
Vitamin K_1	...		76.9
	25		24.6
	50		22.7
	100		19.7
	200		16.1
	400		14.9
	800		14.1
	1,600		12.9
Menadione	7	25	27.3
	26	100	24.1
	105	400	22.5
	419	1,600	20.0
	1,677	6,400	16.1
	3,354	12,800	16.4
Menadione sodium bisulfite complex‡	29	25	25.8
	114	100	25.0
	458	400	18.4
	1,830	1,600	17.6
	7,322	6,400	20.6
	14,643	12,800	21.9

* 400 mg./kg. diet.
† Calculated on the basis of the reciprocals of the individual prothrombin times.
‡ Consists of two thirds of menadione sodium bisulfite.

the reciprocal of the square root, as recommended by Dr. H. W. Norton of The University of Illinois. For prothrombin times, we are using reciprocals and are plotting these, as recommended by several researchers, against the logarithms of the dose of vitamin K given to the animal. I do not know whether these approaches are the best ones feasible, and there is certainly room for further exploration in this area.